

Islet vascularization in Type 2 Diabetes Mellitus

Dissertation

zur Erlangung des Grades eines Doktors der Naturwissenschaften
im Fachbereich Biologie/Chemie der Universität Bremen

vorgelegt von

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Bremen, 07.10.2015

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I. **Abstract**

Diabetes is a complex metabolic disorder characterized by the failure to maintain normoglycemia stemming from dysfunctional islet of Langerhans. It is caused by an autoimmune destruction of insulin secreting β -cells in case of type 1 diabetes (T1D), or non-insulin dependent diabetes, caused by lack on insulin action and production in type 2 diabetes (T2D) and by insulin insufficiency during pregnancy as in gestational diabetes mellitus (GDM). T2D accounts for at least 90% of the cases of diabetes, although it may remain undetected or at a pre-diabetic stage for several years. Thus, therapeutic intervention to prevent the progression to T2D is a major goal to subside the incidence of the disease and thus prevent further metabolic complications.

T2D is most commonly associated with obesity and thus peripheral insulin resistance. In the face of insulin resistance there occurs an array of molecular mechanisms, one of the major activator being inflammation. In serum, adipose tissue, liver and pancreatic islets from T2D patients, pro-inflammatory cytokines like IL-1 β , CXCL10, TNF α , IL-6 have been detected and clinical trials are initiated to prevent inflammatory action. In our study, we showed anti-mouse CXCL10 antibody prevented diabetes progression; it improved glucose tolerance, insulin sensitivity and restored glucose stimulated insulin secretion in the HFD fed mice. CXCL10 antagonism also prevented upregulation of pro-inflammatory cytokines, IL-1 β , IL-6 and CXCL10 mRNA in isolated islets, CXCL10 in adipose tissue and liver of high fat/high sucrose diet (HFD) fed mice.

Dipeptidyl peptidase-4 (DPP-4) inhibitors are oral antidiabetics widely in use for T2D treatment. The first agent of its class – sitagliptin – was approved by the FDA in 2006 and since has been investigated for its direct effects on islet function. The gluco-incretin hormones GIP and GLP-1 secreted by the intestinal endocrine cells potentiate glucose stimulated insulin secretion but are rapidly inactivated by DPP-4. We treated cultured human islets with a diabetic milieu of high glucose, palmitate, cytokines and H₂O₂ in presence of the DPP-4 inhibitor linagliptin. Linagliptin restored β -cell function and turnover, via mechanism involving stabilization of secreted GLP-1 in islet supernatants. Obesity induced insulin resistance requires expansion of β -cell mass to maintain normoglycemia. There occurs a compensatory islet hyperplasia,

progressing with altered islet vascularization and possibly angiogenesis, inflammation and eventually leading to reduced β -cell mass, β -cell failure and hyperglycemia. The molecular mechanisms involved in the concomitant pathophysiology of islet endothelial cells in T2D has focused on effects mediated by VEGF-A. In this study, we aimed to identify the regulation of and the changes driven by the Angiopoietin/Tie angiogenic factors in islet vascularization and function during the progression of T2D.

Ang-1 expressed by perivascular cells and β -cells and Ang-2 expressed by endothelial cells exert their autocrine and paracrine effects via the cognate receptor Tie-2, on the endothelial cells. Tie-2 signaling maintains quiescent vasculature via constitutive Ang-1 expression whereas Ang-2 is known to be in play in demand for angiogenesis or pathological stimuli involving inflammation. Ang/Tie regulation and thus its role in diabetes and islet vascularization is so far poorly understood.

Islet vessel area was increased in autopsy pancreases from T2D subjects, compared to controls. Vessel markers Tie-1, Tie-2 and CD31 were upregulated in mouse islets upon HFD feeding from 8 to 24 weeks. Ang-2 was transiently upregulated in mouse islets at 8 weeks of HFD as well under gluco-lipotoxicity in vitro in human and mouse islets, in contrast to its downregulation with cytokine treatment. Ang-1 on the other hand was oppositely regulated, with reduction under glucolipotoxic conditions and upregulation by cytokine milieu. Modulation of such changes in Ang-2 expression by its overexpression or the inhibition of its receptor Tie-2 impaired β -cell function at basal conditions but protected islets from cytokine induced apoptosis in vitro. In vivo, β -cell-specific Ang-2 overexpression in mice induced vascularization under normal diet but contrastingly hypovascularized islets under HFD together with increased apoptosis and reduction of β -cell mass.

Our data show that increased islet hypervascularization is paralleled with T2D. Maintaining physiological Ang-2 levels is important for islet vascularization and β -cell survival.

II. Zusammenfassung

Diabetes ist eine komplexe Stoffwechselstörung, gekennzeichnet durch das Versagen der Langerhans'schen Inselzellen, Normoglykämie aufrecht zu erhalten. Dies wird vermutlich im Fall von Typ-1-Diabetes (T1D oder nicht-insulin abhängiger Diabetes) durch eine autoimmune Zerstörung der Insulin sekretierenden β -Zellen ausgelöst; durch einen Mangel an Insulin sensitivität und –produktion im Fall des Type-2-Diabetes (T2D), und durch Insulin insuffizienz während Schwangerschaft beim Schwangerschafts diabetes (GDM). T2D betrifft mindestens 90% der Fälle von Diabetes, obwohl er über mehrere Jahre unerkant oder in einem prädiabetischen Stadium verbleibt. Daher ist die therapeutische Intervention von extremer Wichtigkeit, um Ausbruch und Fortschreiten von T2D und dessen schwere metabolische Komplikationen zu verhindern.

T2D steht im Zusammenhang mit Übergewicht und einer peripheren Insulinresistenz. Im Falle einer Insulinresistenz treten eine große Anzahl molekularer Mechanismen auf, die durch subklinische Entzündungen aktiviert werden. Bei T2D Patienten wurden im Serum, im Fettgewebe, Leber und auch in den pankreatischen Inseln pro-inflammatorische Zytokine wie IL-1 β , CXCL10, TNF α und IL-6 gefunden. Klinische Studien wurden initiiert, diese Entzündungsmarker und dessen Wirkung zu inaktivieren. In unserer Studie konnten wir zeigen, dass der anti-Maus Antikörper CXCL10 ein Fortschreiten von Diabetes verhindert; er verbessert die Glucosetoleranz, die Insulinsensitivität und stellte die Glucose-stimulierte Insulinsekretion bei mit fett- und zuckerreiche Diät (HFD) gefütterten Mäusen wieder her. Der CXCL10 Antagonist verhinderte zudem eine Hochregulierung von pro-inflammatorischen Zytokinen, IL-1 β , IL-6 und CXCL10 mRNA in isolierten Inseln, sowie von CXCL10 in Fettgewebe und Leber von HFD gefütterten Mäusen.

Dipeptidyl-Peptidase-4 (DPP-4) -Hemmer sind orale Antidiabetika, welche weit verbreitet im Einsatz für T2D Behandlung sind. Das erste Mittel seiner Klasse - Sitagliptin - wurde von der FDA im Jahre 2006 zugelassen und wird seitdem auf seine direkten Auswirkungen auf die Inselfunktion untersucht. Die Inkretin hormone GIP und GLP-1 werden durch die intestinalen endokrinen

Zellen sezerniert und potenzieren Glucose stimulierte Insulinsekretion, werden aber schnell durch DPP-4 inaktiviert. Wir behandelten isolierte humanische Inselzellen mit einem diabetischen Milieu bestehend aus Glucose, Palmitat, Zytokinen und H₂O₂, in Gegenwart des DPP-4-hemmers Linagliptin. Linagliptin stellte die β -Zellfunktion und Überleben durch die Stabilisierung von sezernierte GLP-1 in den Überständen von kultivierten Inseln wieder her. Eine durch Fettleibigkeit induzierte Insulinresistenz erfordert eine Erhöhung der β -Zellmasse, um Normoglykämie zu erhalten. Um den erhöhten Insulinbedarf auszugleichen, kommt es zur Inselhyperplasie, mit veränderter Gefäßbildung in den Inseln und möglicher Angiogenese und Entzündungen, welche letztendlich zu einer reduzierten β -Zellmasse, β -Zellversagen und Hyperglykämie führen. Die molekularen Mechanismen, die gleichzeitig in der Pathophysiologie der Endothel- und Inselzellen in T2D beteiligt sind, werden teilweise durch den Vasculären Wachstumsfaktor VEGF-A vermittelt. Ziel dieser Arbeit war es, die Regulierung der Insel-Vaskularisierung und Funktion und die durch die angiogenen Faktoren Angiopoietin/ Tie bedingten Veränderungen während des fortschreitenden T2D zu untersuchen.

Ang-1, exprimiert in perivaskulären und β -Zellen, sowie das in Endothelzellen exprimierte Ang-2, üben ihre autokrine und parakrine Wirkungen über den zugehörigen Rezeptor Tie-2 auf Endothelzellen aus. Der Tie-2-Signalweg stabilisiert ein physiologisches Blutgefäß system durch konstitutive Ang-1-Expression, wohingegen Ang-2 eine Rolle bei Angiogenese und in pathologischen Veränderungen, wie bei Entzündungen spielt. Über die Ang/ Tie Regulierung im Diabetes und deren Einfluss auf die Insel-Vaskularisierung ist bisher wenig bekannt.

Meine Untersuchungen zeigen, dass das Blutgefäßvolumen in den Inseln im T2D Pankreas im Vergleich zu nicht diabetischen Kontrollen erhöht ist. Die Gefäßmarker Tie-1, Tie-2 und CD31 waren in Maus-Inselzellen nach 8-24 Wochen HFD Fütterung verstärkt. Ang-2 war nach 8 Wochen HFD Fütterung in Maus Inselzellen, sowie *in vitro* unter glucolipotoxischen Bedingungen in humanen und Maus Inselzellen erhöht, wohingegen es durch eine Zytokin-Behandlung vermindert wurde.

Der Gegenspieler Ang-1 wurde entgegengesetzt reguliert, mit Verminderung unter glucolipotoxischen Bedingungen und Hochregulation im Zytokinmilieu.

Eine Veränderung der Ang-2-Expression durch Überexpression oder die Inhibierung des Rezeptors Tie-2 führte zu einer Beeinträchtigung der β -Zellfunktion unter basalen Bedingungen, schützte die Inseln aber vor Zytokin induzierter Apoptose *in vitro*. *In vivo* induzierte eine β -Zell-spezifische Ang-2-Überexpression in Mäusen bei normaler Ernährung keine veränderte Vaskularisierung, jedoch wurde in den HFD Mäusen eine Ang-2 induzierte Hypervaskularisierung der Inseln, zusammen mit erhöhter Apoptose und Reduktion der β -Zellmasse beobachtet.

Unsere Daten zeigen, dass eine erhöhte Insel-hypervaskularisierung mit der Entwicklung von T2D einhergeht. Die Aufrechterhaltung der physiologischen Ang-2 Level ist entscheidend für die Insel-Vaskularisierung und das Überleben der β -Zelle.

III. Abbreviations

ADP	Adenosine diphosphate
Ang	Angiopoietin
ATP	Adenosine triphosphate
CD	Cluster of differentiation
DPP-4	Dipeptidyl peptidase-4
EC	Endothelial cell
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FFA	Free fatty acids
FGF	Fibroblast growth factor
FTO	Fat mass and obesity
GAD	Glutamic acid decarboxylase
GDM	Gestational diabetes mellitus
GIP	Gastric inhibitory polypeptide
GK	Goto-Kakizaki
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
GSIS	Glucose stimulated insulin secretion
HFD	High fat diet
HUVEC	Human umbilical vein endothelial cells
IA-2	Islet antigen-2
IAA	Insulin autoantobodies
ICA	Islet cell autoantibodies
ICSA	Islet cell surface autoantibodies
IFN- γ	Interferon- γ
IL-1 β	Interleukin 1 β

IPGTT/ITT	Intraperitoneal glucose/insulin tolerance test
MCR4	Melanocortin receptor 4
ND	Normal diet
NOD	Non-obese diabetic
PC 1/3	Prohormone convertase 1/3
PECAM	Platelet endothelial cell adhesion molecule
RT-PCR	Real-Time Polymerase chain reaction
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TCF7L2	Transcription factor 7 like-2
TGF	Tumor growth factor
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor- α
VDF	Vancouver diabetic fatty
VEGF	Vascular endothelial growth factor
WHO	World health organization
ZDF	Zucker diabetic fatty
ZnT8	Zinc transporter 8

A. Introduction

This doctoral thesis mainly focuses on the role of islet vascularization via Angiopoietin/Tie angiogenic factors in maintaining β -cell survival and function and thus, progression to T2D. Other two parts aimed to study, anti-inflammatory therapy in T2D, via CXCL10 antagonism and role of the DPP4 inhibitor, linagliptin on cultured islet survival and function.

1. Diabetes

Diabetes is a complex metabolic disorder to affect about 347 million people by 2014 worldwide according to WHO statistics and this number is predicted to double by 2030, with diabetes being the 7th leading cause of death. The most common symptoms for diabetes include excess urination (polyuria), excess thirst (polydipsia), unusual weight loss, fatigue, irritability and blurry vision. A diagnosis of 126mg/dL or higher fasting blood glucose levels are used to determine diabetes. Glycated haemoglobin, HbA_{1c} levels determination is a more reliable test than blood glucose and value of $\geq 6.5\%$ (48 mmol/mol) is considered diagnostic for diabetes. Diabetes types 1, 2 and 3 are classified based on the cause, type 1 (earlier known as insulin-dependent diabetes) is caused by autoimmune destruction of β -cells, type 2 (insulin independent diabetes) results from impaired insulin secretion and action and type 3 (gestational diabetes) develops during pregnancy due to failure to compensate for the increase in insulin demand.

1.1 T1D

Type 1 diabetes accounts for only 10% of the incidence of diabetes, it is much more dramatic and the mortality rates are much higher than other forms of diabetes. An absolute deficiency in insulin occurs due to a T-cell-mediated autoimmune destruction of the β -cells of the pancreas [1]. Although the main cause of this destruction is attributed to genetic factors, environmental factors like viruses are also involved. Most common incidence of T1D is among children and adolescents, although all age groups may be affected. More recently, the younger population acquires the disease in correlation with obesity [2].

The disease develops as a result of a long moderate symptomless phase wherein the islet leukocyte infiltration occurs and chronic inflammatory response is elicited. This is followed by an acute diabetic phase with

absolute insulin deficiency T-cells have been found to respond to several autoantigens including peptides derived from insulin and glutamic acid decarboxylase (GAD) and killing β -cells [3]. T1D immune markers involve a list of autoantibodies against such antigens as islet cell (cytoplasmic) autoantibodies (ICAs), islet cell surface autoantibodies (ICSAs), insulin autoantibodies (IAAs), islet antigen-2 (IA-2A) and zinc transporter 8 (ZnT8) [4]. The initial steps involve presentation of β -cell autoantigens to $CD4^+$ T-helper cells on MHC class II molecules by macrophages and/or dendritic cells. The activated macrophages and $CD4^+$ T-cells then release cytokines including interleukin (IL)- 1β , tumor necrosis factor (TNF)- α and interferon (IFN)- γ , that attracts and activate $CD8^+$ T-cells in the islets which subsequently destroy β -cells by mechanisms like release of granzymes and perforin as well as by Fas-Fas-Ligand interactions [5].

1.2 T2D

The major cause of type 2 diabetes is undoubtedly over nutrition and a sedentary lifestyle, leading to obesity. Obesity induced insulin resistance and the resultant/causative β -cell failure leading to hyperglycemia is a hallmark of type 2 diabetes.

Obesity induced insulin resistance involves several stages to T2D progression [6]. The foremost criteria being, peripheral tissue unresponsiveness, mainly proposed to be caused by mechanism post-insulin receptor signalling, probably via the insulin receptor substrates. This increases the demand in insulin and causes a β -cell mass expansion. The initial compensation in demand of increased insulin is fulfilled by simply higher insulin secretion and this is followed by an expansion of β -cell volume or mass by proliferation. The nutritional overload in-turn causes a β -cell overwork and often β -cell failure due to metabolic or oxidative stress and additionally an inflammatory response, leading to β -cell death [7, 8].

Such β -cell failure most likely occurs through genetic predisposition [9]. The heritability of the disease is estimated to be 40-80% of the total susceptibility accounting for the cause of T2D. No single gene may be responsible for this heritability but the predisposition may be polygenic in

nature. The genes may be directly involved in insulin secretion, e.g. *KCNJ11* – encoding part of β -cell K^+ -ATP channel, or insulin action [10], *IRS-1* – insulin receptor substrate or β -cell proliferation [11]. A recently published genome-wide association study of obesity related traits shows fat mass and obesity-associated (*FTO*) gene, melatonin receptor 4 (*MCR4*) and transcription factor 7-like 2 (*TCF7L2*) having the strongest association with the disease [12].

1.2.1 Islet inflammation in T2D

T2D onset is clearly seen only when β -cell failure occurs in the face of insulin resistance though the initial stages of β -cell functional impairment have not been widely investigated. More and more evidence suggest a role for chronic islet inflammation playing a significant role in β -cell dysfunction and failure in T2D [13-15] (Fig.1). An elevated number of islet associated macrophages are seen with increased expression of IL-1 β and depletion of these macrophages improves glucose tolerance in high fat diet fed mice. IL-1 β antagonism, by a monoclonal antibody improves glycemia and β -cell function in diet induced obesity [16], reduced hyperglycemia and tissue inflammation in GK rat [17]. Elevated levels of IL-6 and IL-1 β are seen in islets by high fat diet feeding [18]. Clinical trials targeting inflammation in T2D, mainly via IL-1 β blockade, showing glycemic control and no adverse effects with drugs, have now gained momentum [19]. TNF antagonism is also has also shown positive results although lack of long-term treatments are lacking to prove its beneficial role in T2D prevention at the moment.

The role of chemokines involved in T2D progression also comes from studies that found elevated levels of CXCL10/IP-10 in plasma associated with type 2 patients [20]. In the face of hyperglycemia and hyperlipidemia a strong peripheral tissue inflammation is elicited, with TLR4 being one of the major immune receptors involved in the adipose tissue and liver [21, 22]. Elimination of the receptor showed attenuation of a pro-inflammatory state in diabetes [23]. CXCL10 was shown to impair mouse and human islet function and induce β -cell apoptosis via the TLR-4 [24]. In this study, we have used an antibody to CXCL10 in mice kept on high fat/ high

sucrose diet feeding for 16 weeks and investigated effects on glycemia and peripheral tissue inflammatory status (manuscript in appendix).

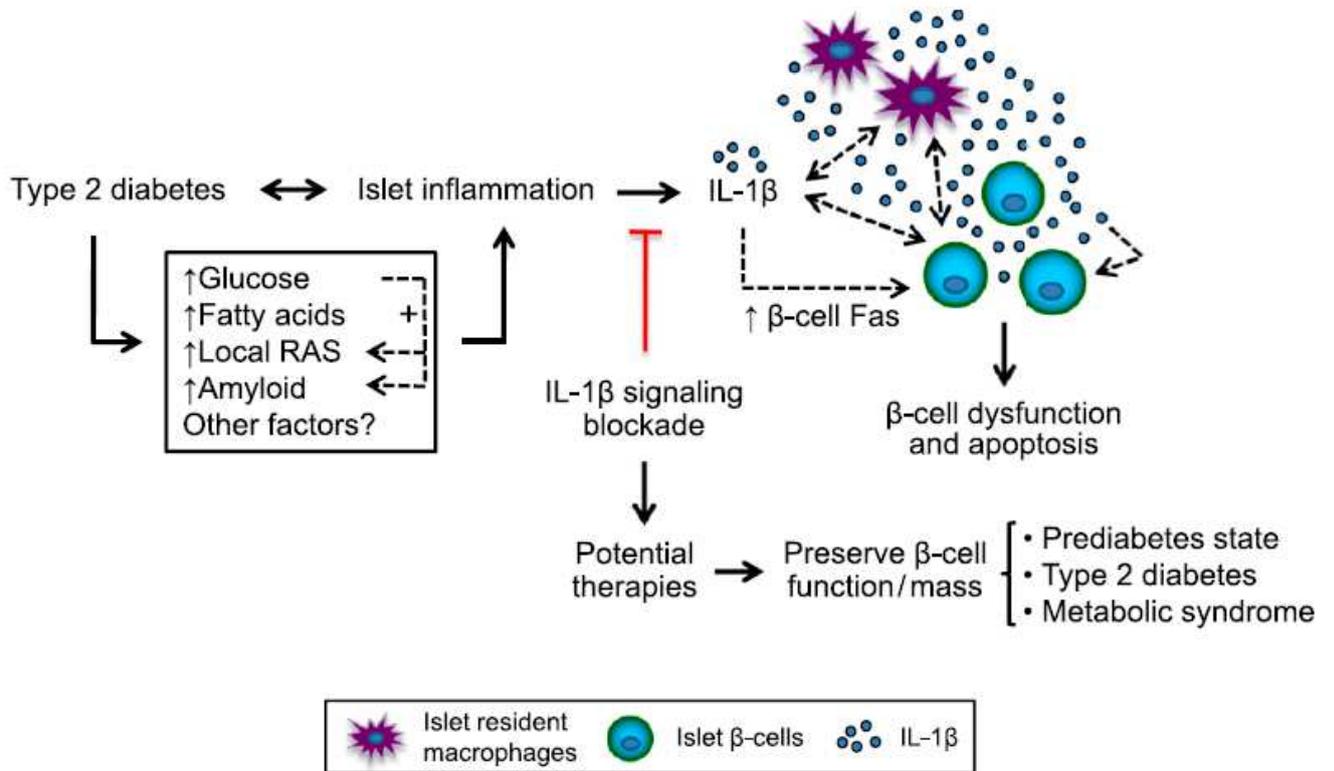


Figure 1: Proposed mechanism of IL-1β in islet inflammation in T2D

Glucose and lipotoxicity, and deposition of islet amyloid induce expression of IL-1β and islet associated macrophages in T2D. This in turn induces β-cell dysfunction and apoptosis. IL-1β blockade improves glycemia and preserved islet function by abrogating β-cell apoptosis and maintaining β-cell mass [25].

Although, T2D is characterized by interplay of several organs and secreted factors in its progression the β-cell failure remains central in its ultimate cause. Thus, it is important to look at the islet structure and anatomy.

1.3 Pancreatic islet structure and vascularisation

The pancreatic islets of Langerhans comprise the endocrine region, one part of the glandular organ - pancreas; with the exocrine region forming the other part (Fig.2a,b). The exocrine portion is mainly composed of acinar cells and is responsible for the secretion of digestive enzymes like trypsinogen and chymotrypsinogen. The islets of Langerhans can be described as highly vascularised mini-organs made of the following endocrine cells and their hormones – α-cell; glucagon, β-cell; insulin, δ-cells, somatostatin, PP-cells;

pancreatic polypeptide and ϵ -cells; ghrelin (Fig.2). The islets comprise of 1-2% of the total pancreatic mass but are provided with 10-15% of the blood supply [26]. Thus, they are in close connection with the endothelial cells and the surrounding extracellular matrix, forming a specialized capillary network serving the tight regulatory role of islets in glucose sensing and homeostasis (Fig.2c,d). The islet function is regulated by neural, paracrine as well as endocrine signals and the islet vascular system maintains the intercellular communication between the endo and exocrine pancreas and to multiple tissues.

A peek into the vascular anatomy of the pancreatic islet reveals that each islet is supplied by 1-5 arterioles branching into capillaries forming a spherical framework like that of a glomerulus [27]. Differences in mouse and human islet cell- and angio-architecture have remained controversial despite several morphometric analyses. The issues at hand have been the proportion of β -cells in islets, their mantle/core distribution within the islets and lastly the proximity of different cell types to the blood vessels. A recent study by Bonner-Weir et al [28], elegantly re-assessed what is known about the islet morphology and added their observation; that blood vessels penetrate non-random clustered β -cells at the islet core in human islets. They also dismiss any major differences in mouse and human islet architecture, although the composition may vary within a pancreas. Islet microvasculature holds significant importance to functional compartmentalization of islet depending on the blood flow [29]. This, direction of the blood flow could determine the intraislet interactions. As far as islet microvasculature is concerned there are two schools of thought, one stating a lack of mantle/core distribution of insulin, glucagon or somatostatin immunoreactive cells and thus, the vessel penetration also being random and not β -cell related [30, 31]. The other conclusion being, β -cells occupy a core position and α -cell at the mantle, with islet vessels penetrating the islet having a close proximity to cells at the core [32]. Bonner-Weir *et al.* report a rosette pattern of β -cells falling adjacent to blood vessels recently described in human islets and previously in rat islets [28, 33].

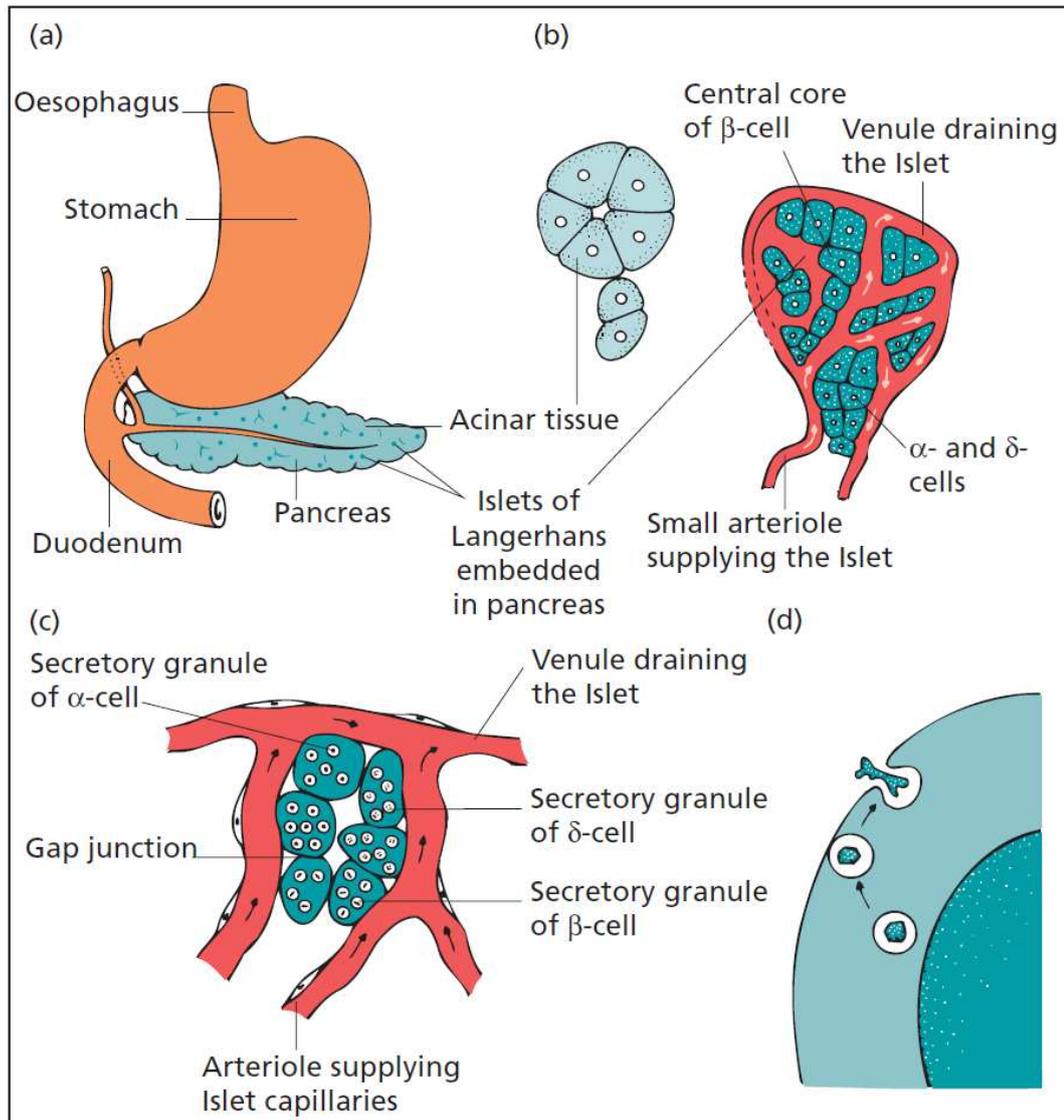


Figure 2: Pancreas and islet vascularisation

(a) The pancreas, located beneath the stomach, connected at the duodenum. (b) Islets of Langerhans, small clusters embedded in the exocrine acinar tissue. Islets mainly constituted of β -cell core, and α - and δ - cells, supplied with one or more small arterioles that then break into capillaries. (c) The endocrine cells are closely connected to capillaries with only a thin basement membrane separating them. The distinct secretory granules enable easy release into the blood stream. (d) Part of β -cell showing secretory granules exocytosis [34]

1.4 Islet function: Insulin secretion and action

β -cells constitute the majority (65-80%) of the islet cells and secrete insulin in response to glucose which increases uptake and storage of glucose, fatty acids and amino acids by its actions on liver, muscle and adipose tissue. It also inhibits glucose production in the liver. Insulin is a 51 amino acid peptide hormone which is initially synthesized as preproinsulin on the ribosomes of rough endoplasmic reticulum and processed into proinsulin, which further breaks down to insulin and C-peptide [35](Fig.3). Insulin is secreted by β -cells in response to glucose in a pulsatile biphasic pattern with its release into the portal vein [36]. Glucose enters the β -cell via GLUT2 transporters and is phosphorylated by glucokinase and enters glycolysis. The increased rate of glucose catabolism raises [ATP/ADP], causing the closing of ATP-gated K^+ channels in the plasma membrane[37]. The reduced efflux of K^+ depolarizes the membrane, thereby opening voltage-sensitive Ca^{2+} channels in the plasma membrane. The resulting influx of Ca^{2+} triggers the release of insulin by exocytosis. Persistent glucose metabolism in the β -cells generates a number of intracellular signalling cascades which then in turn stimulate insulin gene transcription and mRNA translation. Stimuli from the parasympathetic and sympathetic nervous systems also stimulate and inhibit insulin release, respectively. Although intracellular Ca^{2+} seems central in insulin exocytosis, there are physiological instances when there is no further rise in Ca^{2+} but insulin secretion rises, in part due to incretins like acetylcholine, GLP-1, GIP or free fatty acids.

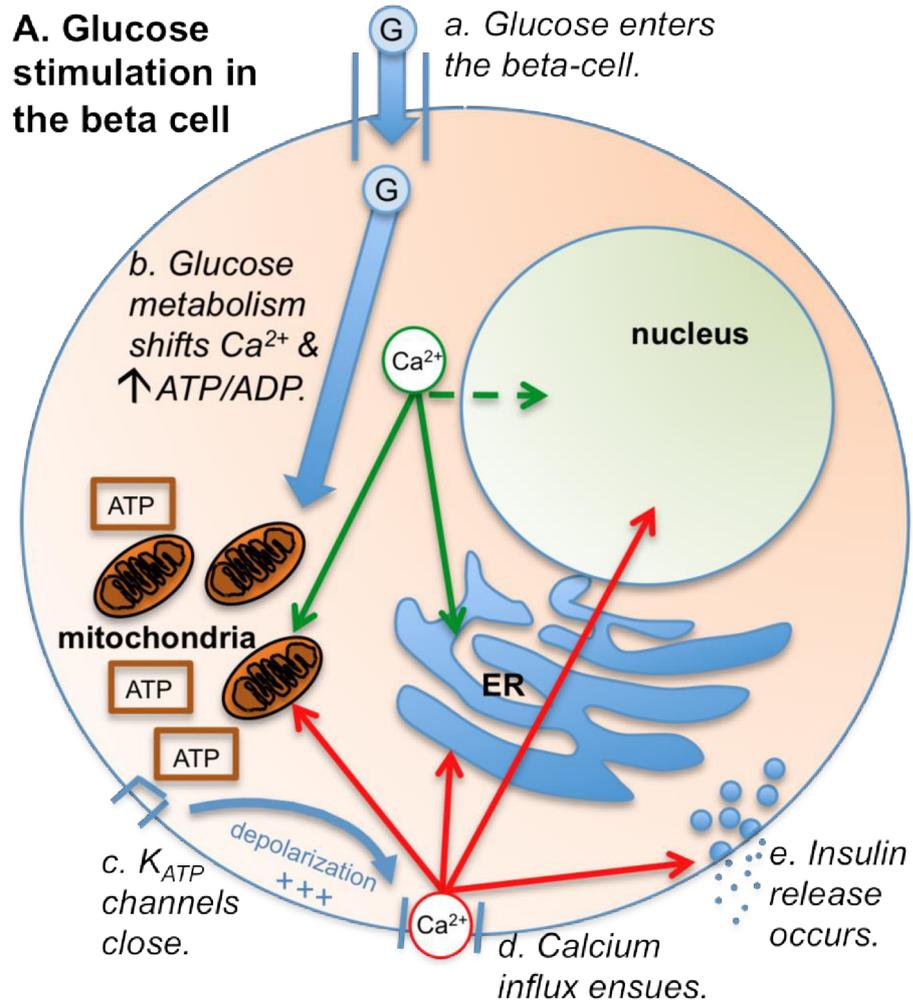


Figure 3: Insulin synthesis and secretion

Uptake and metabolism of glucose leads to elevation in the ATP: ADP ratio, which causes the potassium channels to shut, leading to membrane depolarization and influx of calcium which aids in the exocytotic release of insulin from its storage granules [38]

1.4.1. Incretins in insulin secretion

The gluco-incretin hormones GIP and GLP-1 are secreted by the intestinal endocrine cells and have well established effects on potentiation of glucose stimulated insulin secretion [39](Fig.4). In addition, extrapancreatic actions of GIP and GLP-1 are important in physiological regulation of glucose and energy homeostasis. GLP-1, but not GIP is known to maintain this potentiating capacity even in patients with type 2 diabetes and has been a major therapeutic target since. Several GLP-1 analogues or inhibitors of the enzyme that degrades it are now commonly used in the clinic and ever evolving. GLP-1 and GIP both have also β -cell differentiating [40], proliferating [41] and protect from cytokine induced β -cell apoptosis [42]. GLP-1 or exendin-4, a long-acting agonist both increased β -cell mass in pancreatectomized rats [43], db/db mice had improved glycemia with reduced HbA1c [44] and protected from diabetes with increased pancreatic insulin content in STZ injected rats [45]. GLP-1 or exendin-4 treatment has been shown to prevent or at least markedly reduce each form of β -cell apoptosis; TNF α induced [15], under glucolipotoxicity [13] or under ER stress by activating the unfolded protein response [46].

GLP-1, in its biologically active form is lower than the total GLP-1 found in the plasma, and is just 10-20% [47]. This indicates that GLP-1 is a high affinity substrate for a ubiquitous exopeptidase dipeptidyl peptidase-4 (DPP-4) DPP-4 inhibitors are now a well established line of drugs in the clinic for diabetic medications. Beneficial effect of DPP-4 inhibitors are not only restoring normoglycemia [48] but also restores β -cell survival and neogenesis in the rat streptozotocin model [49] as well as in the VDF Zucker rats [50].

We investigated the mechanism of direct effects of the DPP-4 inhibitor, linagliptin on β -cell survival and function in isolated human islets (appendix) [51].

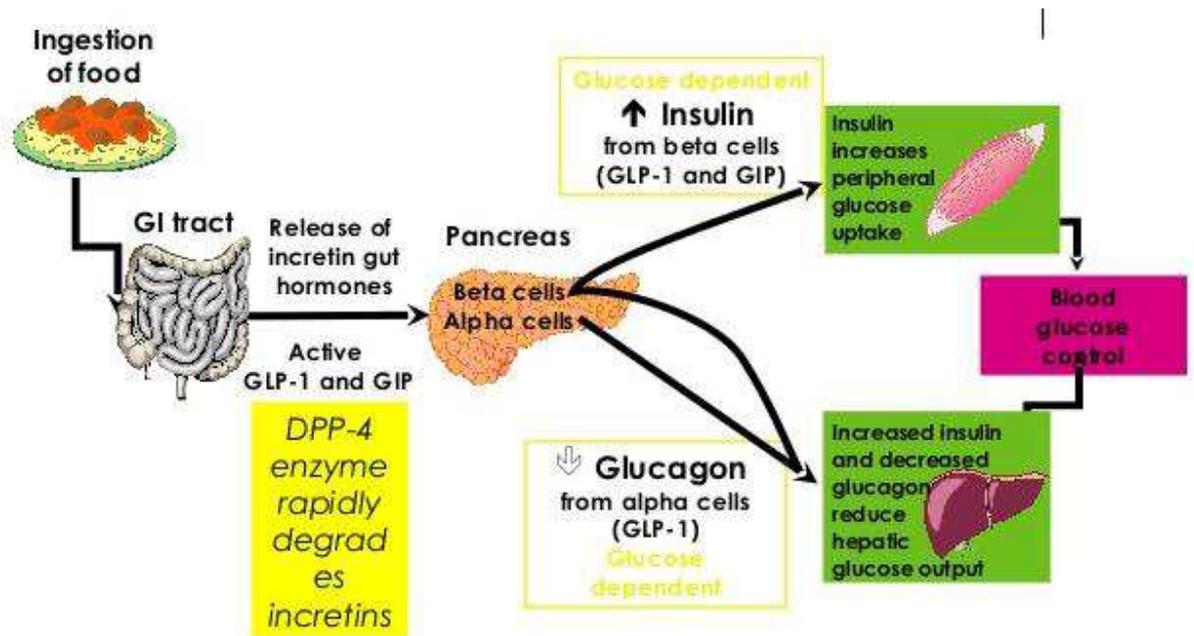


Figure 4: Incretins and glycemic control

GLP-1 and GIP are released by intestinal cells upon ingestion of food, which then act on the beta-cells to potentiate glucose-stimulated insulin secretion which regulates peripheral glucose uptake in the skeletal muscle and reduces hepatic glucose production. DPP-4 is a ubiquitously expressed enzyme which acts on GLP-1 and GIP to inactivate them, lowering GLP-1 insulin secretion efficacy. Adapted from [52].

Islet vasculature forms a functionally and structurally significant part of the islet indicating a need for a good understanding of its regulation in healthy and diabetic conditions. Thus, we will now look at the relevant aspects of the vascular system and focusing on its significance for the islets.

2. Vascularisation

The vascular system is an organ system consisting of blood and lymphatic vessels - the arteries, veins and capillaries, carrying oxygen and nutrients to and from the tissues. The blood vessels are formed by the cellular lining of endothelial cells, that don't merely form a barrier between the blood circulation and target tissues but are now known to be involved in a vast range of signalling activities. The vascular system is thus a multifunctional system, playing a vital role in physiology and pathology.

Vascularisation comprises of two distinct processes – vasculogenesis; the formation of new blood vessels from primitive stem cells, that occurs mainly in the early embryogenesis, and angiogenesis; the formation of vessel from pre-existing capillaries. It may occur by sprouting; where mature ECs proliferate and migrate to form new vessels or intussusceptive, occurs by splitting vessels by invasion of interstitial tissue [53] (Fig.5a,b). Though the blood vessels on the whole play a common role in the wide spectrum of tissues, each organ vasculature is characterised by a specialized structure and function. This structure and function define the blood vessels; whether they have a vascular, lymphatic, large vessel or a capillary fate. The arterial/venous character of endothelial cells is decided early during embryogenesis by genetic factors whereas angiogenesis is regulated mostly by physiological stimuli and its molecular signalling cascade [54] (Fig.5c,d).

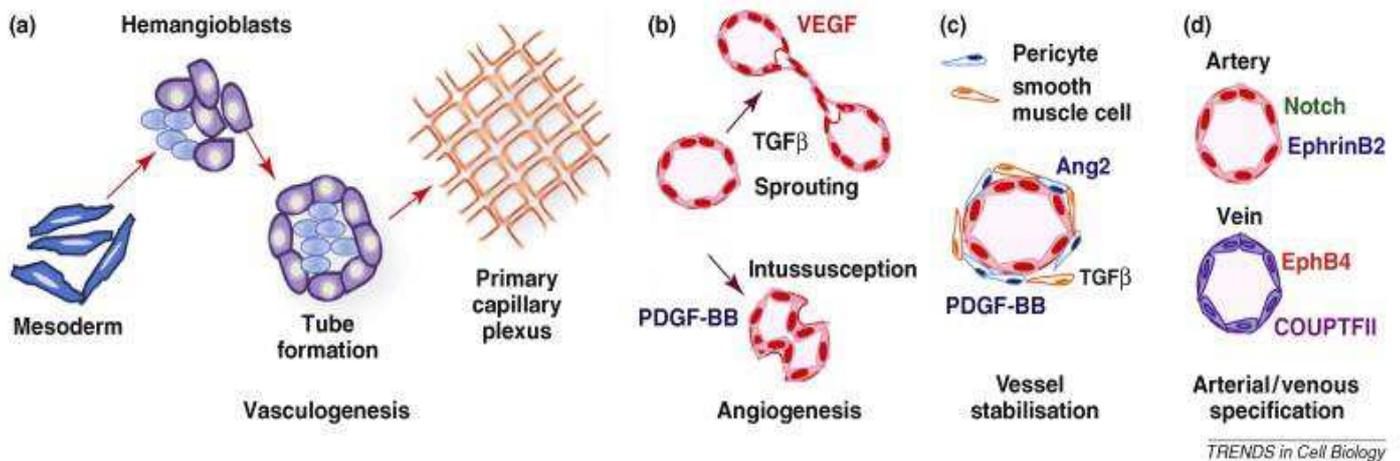


Figure 5: Development of the vascular system and angiogenesis

Vessels are formed by vasculogenesis during the embryonic development from the mesoderm to form the primary plexus (a) Angiogenesis may occur by 2 types; sprouting or intussusception by mainly VEGF or TGF β (b) Pathologies cause vessel reorganization involving pericytes and Ang-2/Tie-2 and PDGF-BB (c) Arterial and venous fate depend on expression of and regulation by Notch and ephrin pathways [55]

2.1 Blood vessel structure and heterogeneity

The blood vessel consists of distinct layers of cells and membranes; the internal endothelium is covered by a basement membrane, created by various matrix proteins secreted by the endothelial cells. The basement membrane is covered by smooth muscle cells sandwiched between elastic membranes in thicker vessels. Blood vessel structure is acutely regulated by the innervations surrounding all these layers and is mainly responsible for modulation of organ blood flow. The capillaries that are closest to contact with specific organ cells consist of the endothelium and a layer of pericytes, which together creates the extracellular matrix surrounding the capillary.

Capillary endothelial cell structure and function differ depending on the environmental cues, various metabolites, growth factors and cytokines, oxygen tension and mechanical forces. [54]. ECs of different organs contain not only distinct subcellular structures, but are also metabolically and physically dynamic regulating blood flow, vessel permeability, thrombosis, thrombolysis, angiogenesis and immunity [56]. Another proof of endothelial heterogeneity among tissues is described by differences in their gene expression and antigen composition [57].

2.1.1 Morphological heterogeneity of the endothelium

Endothelium is continuous or fenestrated, fenestrae (with or without diaphragm), caveolated or with transendothelial channels [58]. The continuous and non-fenestrated endothelia lining the blood and lymph vessels, capillaries (of skin, lungs, kidney, muscles, brain) and chambers of the heart form an uninterrupted barrier between blood and tissues. Continuous fenestrated endothelium on the other hand, with pore sizes ~70 nm are organised in planar clusters and are a characteristic of capillaries of the endocrine gland (pancreas, pituitary etc.), digestive tract mucosa and peritubular part of the kidney. The discontinuous endothelium of the liver sinusoidal endothelial cells, spleen and bone marrow, lack a basement membrane and are instead caveolated.

2.1.2 Heterogeneity in phenotype and markers

Apart from morphological differences, post-capillary endothelial cells express specific markers depending on the vascular bed in which they dwell [54]. For example, lung endothelial cell adhesion molecular-1 is in lung ECs, tPA is highest in brain and glomerular, sinusoidal and lymphatic ECs are negative for vWF [59]. Even PECAM-1, the most frequent EC marker, which controls neutrophils and their migration as well as endothelial monolayer stabilisation, is also expressed on trophoblasts and monocytes. VE-cadherin is restricted to arteries, arterioles and capillaries, with extremely low expression in venous ECs[60].

In addition to their heterogeneous morphology and marker expression, ECs display an enormous diversity in function, making them indispensable in organism homeostasis. Quiescent endothelium presents anti-adhesive, -coagulant and vasodilating properties in contrary to activated ECs, which in addition have elevated transmigration of immune cells[61]. One of the distinguishing roles of activated ECs is their function as mediator of the inflammatory cascade. Endothelial cell permeability dysfunction is a consequence of a severe inflammatory state, often leading to oedema. ECs form the first barrier for immune cell infiltration and thus play an important role in adaptive and innate immunity. Immune cell recruitment is mediated by VEGF and Ang, via cytokines like TNF α , IL-6 and IL-1 β [62]. Further, leukocyte attachment and rolling are mediated by upregulation of P-selectins,

and transmigration by E-selectin. Integrins and endothelial cell adhesion molecules (ICAM-1 and VCAM-1) are then upregulated and the final transendothelial migration is supported by disassembly of the VE-cadherin complex at tight junctions [63].

Regardless of the extent and type of organ vascularisation, the vasculature relies on expansion of networks to fulfil the demands in the state of tissue pathology.

2.1.3 Islet endothelial cells

As stated above, there exists great endothelial cell heterogeneity between capillaries of different organs. Islet endothelial cells are highly fenestrated and surrounded by a basal lamina of no more than 500 nm prior to contact with β -cells [64]. Modulation in islet vasculature via VEGF-A deficiency has shown the crucial role of the high vessel density and fenestrae in controlling the secretory function of islet. One of the first studies showing a direct role of endothelial derived molecules in islet development was by Lammert *et al* [65]. Blood vessel endothelium induces insulin expression in the mouse embryonic tissue *in vitro* and the removal of the dorsal aorta in *Xenopus laevis* embryos failed to induce insulin expression *in vivo*. Yet another proof of endothelial role in islet development was a study showing early dorsal pancreatic development, via induction of pancreatic transcription factor Ptf1a [66]. They also showed influence of endothelial cells on insulin and glucagon gene expression.

Nonetheless, be it simply via the blood flow regulation or intraislet interactions the islet capillary plays a crucial role in islet function and thus glucose homeostasis.

2.2 Islet perfusion and vascularization in diabetes

One of the physiological ways of regulating islet function and secretion is modulation of islet blood flow. Islets are very densely vascularised mini-organs, relying on fine tuning of islet perfusion in demand to insulin. One of the early works of P.O. Carlsson in 1996 demonstrated via microsphere technique that islet blood flow rises with glucose administration without affecting the whole pancreatic blood flow [67]. They also showed such a rise in islet blood flow in pathological situations as in obese *ob/ob* mice

but actually lower blood flow compared to lean controls, when normalised to the islet weight, thus showing poorly perfused bigger islets. This may contribute to the impaired insulin secretion observed in such pathological conditions.

Insulin exerts biological anabolic action on liver, adipose tissue and skeletal muscle affecting glucose, lipid and protein metabolism through insulin receptor signalling and GLUT4 translocation [68]. 60% of the insulin is cleared by the liver via the insulin secretion from the portal vein where its concentration is 3 fold higher than the plasma concentration. Hepatic gluconeogenesis is thus inhibited and glycogen storage promoted. In the skeletal muscle glucose uptake enables glycogen storage and in adipose tissue fat breakdown is suppressed and synthesis promoted. The endothelial insulin receptor signalling was recently shown to directly regulate islet insulin secretion *in vivo* via the insulin receptor substrate-2[69]. There is an increased demand in insulin in response to over-nutrition like in obesity or a lack of insulin occurs in an autoimmune response causing β -cell failure, both eventually leading to chronic hyperglycemia and thus diabetes

Islet microangiopathies were shown in a type 1 diabetic model, alloxan-treated male Wistar rats way back in 1989 with following observations; acellular capillaries, basement membrane thickening, perivascular edema and endothelial cytoplasmic bulging [70]. The same group studied in 1995 the microvasculature in a mouse model of T2D, *db/db* mice [71]. Electron micrographs from these pancreas revealed “capillary scarcity, increase in the mean and diversity of capillary size, pericapillary edema and fibrosis, hypertrophy of pericyte and abundance of actin-like microfilaments and luminal irregularity”, most characteristics indicating hyper-perfusion. Hyperglycemia mediated increase in islet blood pressure was soon discovered in diabetic GK rats by Carlsson *et al*[72]. The islet blood flow or perfusion on the other hand in obese-hyperglycemic (*ob/ob*) mice was increased compared to lean controls but reduced upon correction with islet weight [67]. Both studies implicated a role of islet vessels in the pathogenesis and advancement of diabetes.

It has been long debated whether more vascularisation is better or worse for islet function and the answer seems to be context dependent. Higher blood-perfused native islets with improved function are better revascularised upon transplantation but the very same group of islets are more prone to cellular stress [73]. There are almost equal and opposing views about how islet vessels behave in response to β -cell mass expansion; if islets are hypo or hypervascularised in eventual loss of β -cell failure. Only a recent study by Brissova *et al* [74] have clearly shown a higher islet vessel density in T2D islets associated with amyloid deposition.

90% of all patients with diabetes are affected by type 2 diabetes with cardiovascular disease dependent deaths being responsible for 50-80% of the mortalities. Microvascular complications may occur in patients after acute metabolic distress cause by hyperglycemia, affecting kidneys, eyes and nerves. Macrovascular detrimental effects like myocardial infarction can be seen after a prolonged period of hyperglycemia. Peripheral tissues, adipose and skeletal muscles seem to have lower vessel density in response to obesity [75, 76]. Several studies focusing on angiogenic factors in regulating islet vasculature in diabetes have now brought into focus the underlying mechanism of how islet vessels respond in demand of increased insulin and expansion to β -cell mass. In a HFD mouse model, impaired islet vascularisation was redundant to β -cell mass expansion [77] shown by β -cell VEGF mutant mice. An increase %vessel/islet area upon 4 months of HFD feeding [78] associated with increased VEGF content or no change in vessel density (though vessel area/islet area increased) but dilation in response to β -cell mass expansion [79] in *ob/ob*, *GLUT4^{+/-}* and 4 months HFD fed mice.

VEGF-A is one of most widely studied in context of diabetes, in T1D for islet vascularisation in transplanted grafts and in T2D for islet angiogenesis in response to obesity. VEGF-B improves insulin sensitivity in obesity and anti-VEGF-B therapies are suggested to improve the diabetic phenotype [80].

It remains to be investigated to what extent angiogenesis contributes to β -cell failure and thus progression to T2D.

2.3 Angiogenesis and angiogenic factors

Angiogenesis may be physiological, as in the female reproductive system or pathological. There's a wide range of pathological angiogenesis states from a simple process like wound healing to cardiovascular diseases, diabetes and cancer. The potential of angiogenesis having therapeutic value has stimulated great interest in the past 40 years[81]. One of the major applications of angiogenesis research involves developing anti-angiogenic treatments for tumour growth inhibition [82]. In order to understand the targets of angiogenesis for therapeutic applications we need a thorough understanding of the steps and its molecular mechanisms. Angiogenesis; whether growth or regression, involves a series of well co-ordinated events, with autocrine or paracrine signals on endothelial cells. Hypoxia is one of the key factors initiating angiogenesis to overcome the oxygen depletion and starvation [83]. Angiogenesis is a multi-step process with distinct stages (Fig.6); firstly, the stimulation of the endothelial cells by angiogenic factors. Angiogenic factors classically involved in this process are bFGF, VEGF and Angiopoietins acting on their receptors FGFR, VEGFR and Tie-2, respectively[84-86]. They activate endothelial cells for proliferation, survival and migration. Secondly is the degradation of the basal lamina composed of lamins, collagens and proteoglycans, mainly by matrix-metalloproteinases followed by EC migration and growth in order to form vessel tubes by adhesion molecules like integrins [87]. Remodelling occurs again by a complex interplay of MMPs to dissolve the extracellular matrix. Once, the lumen is formed, the pericytes migrate to the vessel to stabilise them mainly by the Tie-2-angiopoietin-1 signalling and TGF β [88].

In the past decades, FGF, VEGF, HGF, EGF and Angiopoietins have been described to play distinct roles in each step of angiogenesis and pathologies. The VEGF family of angiogenic factors contains more than seven members, with VEGF-A playing the most significant role, binding to VEGFR1 and VEGFR2 receptors regulating angiogenesis and permeability as well as vasculogenesis [84]. On the other hand, Angiopoietin/Tie maintains the quiescent vasculature and endothelial cell survival [86]

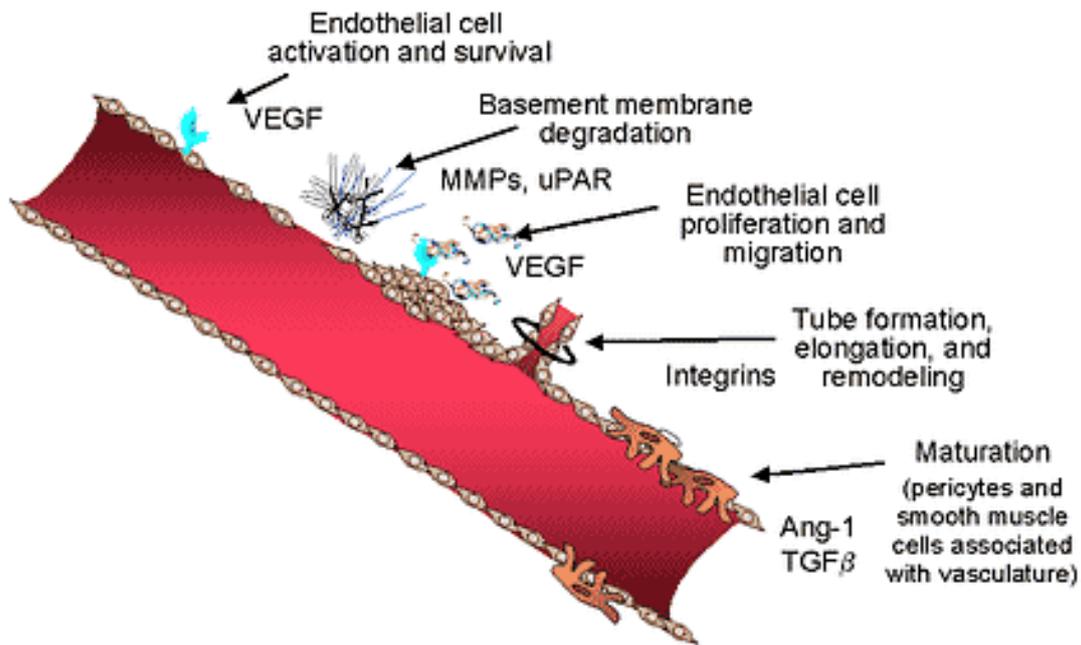


Figure 6: Steps involved in angiogenesis

Angiogenesis initiates with conversion of quiescent EC to activated state and dissociation from pericytes. The MMPs and uPAR signalling dissolved the basal lamina and primes ECs for proliferation and migration. In the next step is the lumen formation regulated by integrins and cell-cell junctions, followed by a final maturation and pericyte migration to the blood vessel tube [89]

2.3.1 Angiopoietin/Tie

The Ang/Tie family of angiogenic factors consists of the receptors Tie1 and Tie2 (also known as TEK) and the ligands angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2) and the mouse/human interspecies orthologs Ang-3/Ang-4, respectively.

2.3.1.1 The Tie Receptors

Tie-1 and *Tie-2* genes, on mouse chromosome 4 encoding the Tie receptors discovered in 1993, were found to be endothelial specific tyrosine kinase receptors of crucial importance to the vascular system[90]. Both the receptors (46% identity) consist of three extracellular epidermal growth factor-like repeats, two immunoglobulin-like domains and three fibronectin type III repeats. The cytoplasmic tail consists of split tyrosine kinase domains. The function of the Tie receptors in vascular integrity and survival was established even before the discovery of their ligands. They were shown to be expressed also on some hematopoietic cell lineages in addition to endothelial cells[91]. The Tie receptors are not required for initial differentiation of endothelial cell

lineage but are essential at later stages for maintenance of the microvasculature. *Tie1* deletion shows normal vascular development upto E13.0 but then causes loss of endothelial cell integrity and death of embryos at E14.5[92, 93]. *Tie2* mutants resemble the *Tie1* mutants but they die earlier at embryonic day 9.5[94]. Though Tie-1 and Tie-2 seem to be functionally similar, Tie-1 is long known as an orphan receptor, but Tie-2 was deorphanized by the discovery of angiopoietins. Tie-1 exerts a weak kinase activity under normal conditions; it does not directly bind angiopoietins but modifies Tie-2 dependent intracellular signalling and can also inhibit Tie-2 ligand binding by Tie-1 ectodomain cleavage [95]. Tie-1 seems to play a role in endothelial inflammatory gene expression [96] Tie-1 exerts proinflammatory properties; its overexpression induces inflammatory gene expression in endothelial cells [97].

2.3.1.2 The Tie Ligands

Angiopoietins are ~75 kDa proteins made up of amino-terminal coiled-coil domain responsible for ligand homo-oligomerization and carboxy-terminal fibrinogen domain containing binding sites for Tie-2 [98]. Ang-1 and Ang-2 have been the most widely studied and Ang-1, mainly expressed by pericytes is the classical agonist of Tie-2, inducing autophosphorylation of the kinase domain and thus regulating intracellular pathways, i.e. cell proliferation, survival, sprouting and migration [99]. Ang-2, expressed mainly by endothelial cells and some smooth muscle cells was classically known to antagonise Tie-2 signalling with a similar affinity as Ang-1. It also induces Tie-1 signalling in a context-dependent manner [100-102]. Ectopic expression of Tie-2 in non-endothelial cells as well as high concentrations of Ang-2 is known to induce Tie-2 signalling activities similar to Ang-1. This was consistent with the finding of Ang-1 [99] and Tie-2 deficient [93] mice having similar phenotypes to Ang-2 overexpressing mice [103]. Unlike constitutive Ang-1 expression, Ang-2 expression is tightly regulated and strongly expressed during angiogenesis and inflammation [104, 105]. It is proposed to regulate angiogenesis in an autocrine manner whereas its inflammatory effects could be paracrine via the immune cells. Ang-1 reduces expression of cell adhesion molecules [106] and Ang-2 on the other hand promotes

vascular leakage and inflammation [107] (Fig.7). Thus Ang-2 upregulation had been correlated to several inflammatory disorders from sepsis to cancer.

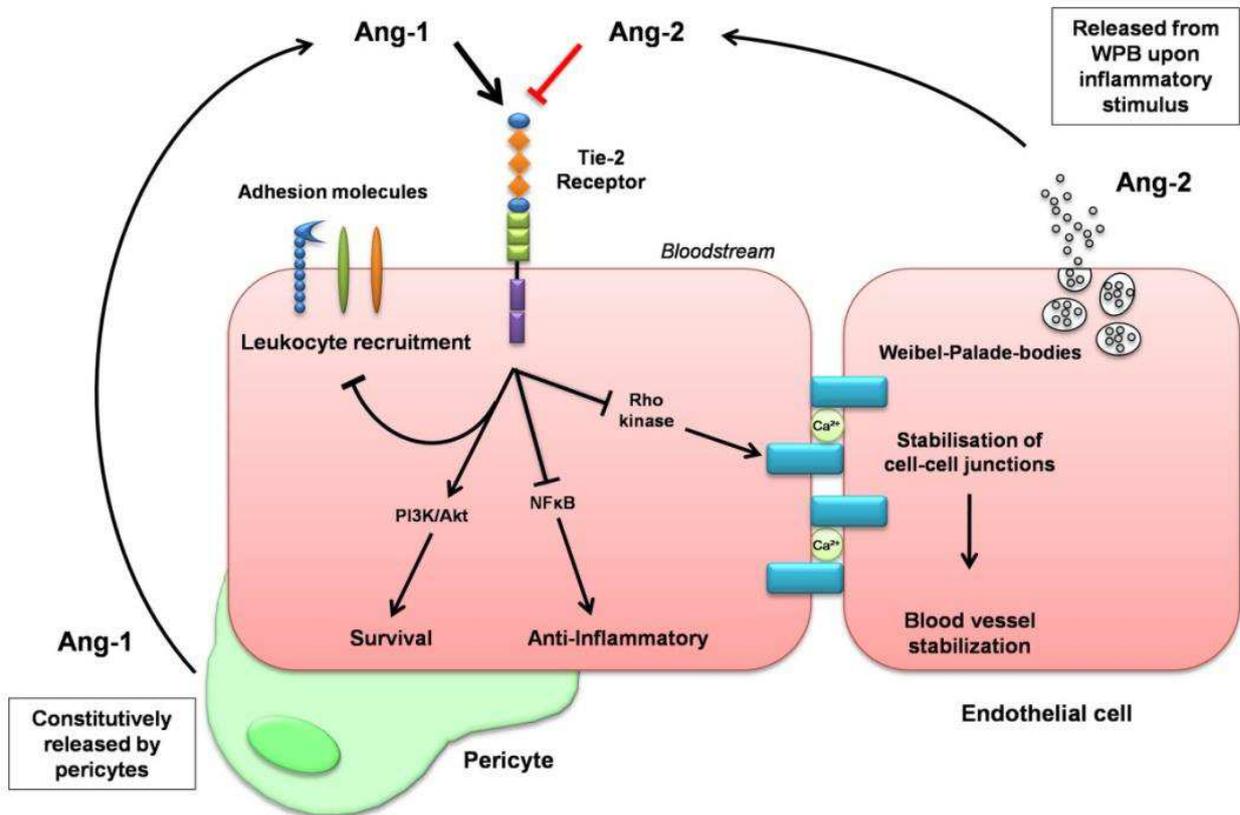


Figure 7: Angiopoietin/Tie-2 signalling

Ang-1 constitutively expressed by pericytes maintains endothelial survival via P13K/Akt and prevents inflammation via NFκB inhibition. Ang-2 is induced upon stimuli like hypoxia and cytokines like TNF, and released from the Weibel Palade bodies to block Ang-1 binding to Tie-2. It induces inflammatory pathways and upregulates cell adhesion molecules and destabilizes the vessel by pericyte dropout www.snipview.org

2.4 Aim of the thesis

The beneficial role of Ang-1 in preventing diabetes-induced vascular complications has been extensively reviewed [108]. Ang-1 protects islets from cytokine induced apoptosis promoting islet function and revascularisation in transplanted islet grafts [109]. Circulating Ang-2 and sTie-2 levels have been associated with diabetic microangiopathies and HbA1c levels [110, 111]. Upregulating Ang-2 in islets transplanted to STZ treated mice was correlated with impaired vascularisation with lower islet vessel density and its inhibition improved glycemia in these mice [112]. Though some studies have shown roles of Ang-2 in islet vascularisation in developing tissue or basal conditions, not much is known about its role in islet vascularisation in T2D.

Our aim was to identify if upregulated levels of Ang-2 impair islet vascularisation in T2D. Using β -cell specific Ang-2 overexpressing mouse model and Ang-2 overexpression and downregulation in mouse and human islets in vitro we found protective and deleterious effects of Ang-2 on β -cell survival and function.

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Angiopoetin-2 signals do not mediate the hypervascularization of islets in T2D

This is a manuscript in preparation and to be submitted after repetition of some
experiments

Angiopoetin-2 signals do not mediate the hypervascularization of islets in T2D

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Running Title

Ang/Tie in islet vascularization in T2D

Keywords

Ang-1, Ang-2, Tie, β -cells, islets, diabetes, apoptosis

Islet angiogenesis has been suggested to influence β -cell survival and function in progression to type 2 diabetes (T2D). Obesity induced insulin resistance requires expansion of β -cell mass to maintain normoglycemia. Failure of the β -cell to compensate for the increased insulin demand as a result of a complex interplay of genetic and environmental factors, e.g. ongoing inflammation in the islets and impaired vascular function, eventually leads to diabetes.

The molecular mechanism involved in the concomitant pathophysiology of islet endothelial cells in T2D is mainly mediated via VEGF-A. In this study, we aimed to identify whether there are changes in islet vascularization during the progression of T2D, focusing on Ang/Tie angiogenic factors.

Islet vessel area was increased in autopsy pancreases from T2D subjects, compared controls subjects. Vessel markers Tie-1, Tie-2 and CD31 were upregulated in mouse islets upon HFD feeding from 8 to 24 weeks. Ang-2 was transiently upregulated in mouse islets at 8 weeks of HFD as well under glucolipotoxic conditions (22.2mM glucose, 0.5mM palmitate) in vitro in human and mouse islets, in contrast to its downregulation with cytokine treatment (IL-1 β , IFN- γ and TNF- α). Ang-1 on the other hand was oppositely regulated, with reduction under glucolipotoxic conditions and upregulation with cytokine milieu. Modulation of such changes in Ang-2 expression by its overexpression or the inhibition of its receptor Tie-2 impaired β -cell function at basal conditions but protected islets from cytokine induced apoptosis.

In vivo, β -cell-specific Ang-2 overexpression in mice induced under normal diet but contrastingly hypovascularized islets under HFD together with increased apoptosis and reduction of β -cell mass.

Our data show that increased islet hypervascularization is paralleled with T2D and that maintaining physiological Ang-2 level is important for islet vascularization and β -cell survival.

Introduction

Pancreatic islets are densely vascularized mini-organs constituting 1-2% of the pancreatic mass but supplied by 5-15% of the pancreatic blood flow [1, 2]. The islets are penetrated by a specialized endothelium with more highly fenestrated capillaries than the exocrine tissue and with a unique basement membrane [3]. The laminin isoforms are known to directly influence β -cell function and proliferation [4]. In their remarkable study Lammert *et al.* showed that the islet endothelium sends inductive signals to direct islet development, beyond merely serving the metabolic demand [5]. This suggests endothelium regulated islet survival.

Obesity induced insulin resistance is characterized by a compensatory increase in β -cell function and mass. Although the β -cell has the remarkable capacity to compensate for the higher insulin demand, it often leads to β -cell overwork and a consequent loss of β -cell function and survival. Consequently, hyperglycemia and type 2 diabetes (T2D) manifests. The role of islet angiogenesis during compensated β -cell mass expansion and function has been highlighted in the ZDF rat, showing a transient islet hypervascularization preceding β -cell failure [6]. Other studies have revealed a dilation of pre-existing islet vessels in response to β -cell mass expansion [7] or an indispensability of proper vasculature during β -cell mass and function adaptation in response to high fat diet induced obesity in mice [8]. In contrast a recent study shows that human islets present a higher vessel density in T2D, which occurs together with amyloid depositions in areas of thicker capillaries [9]. Such higher vessel area in T2D suggests that vessels, although they bring nutrients, which are especially important in the β -cell compensation state, may also be detrimental through the delivery of inflammatory products from the circulation to the proximity of the highly sensitive β -cells. Variations in observation of the physiological relevance of islet vessel density on islet function may be owing to variations in islet angio-architecture within and between species. Human islets were found to have lower vessel density than mouse islets [9] and whether islet capillaries favour proximity to β -cells is yet an evolving debate [10, 11].

Islet angiogenesis is promoted by three major families of angiogenic factors; vascular endothelial growth factors (VEGF), angiopoietins (Ang) and ephrins (Eph). VEGF-A among the VEGFs, is the most highly expressed in the islet and the most extensively studied in islet development and the diabetic patho-physiology. VEGF-A

deficiency leads to a less dense and immature capillary network during embryonic [12, 13] as well as postnatal islet development [14] and reduces insulin content and secretion [15]. VEGF-A overexpression on the other hand, enhances islet vascularization but impairs islet morphogenesis and β -cell proliferation [16, 17]. The Ang/Tie family of angiogenic factors is so far poorly studied in the context of diabetes. It consists of the ligands Ang-1,-2 and Ang-4 (its mouse orthologue Ang-3) and the tyrosine kinase receptors Tie-1 and Tie-2. Ang-1 expressed mainly by the perivascular cells and β -cells in mouse and human islets [14], is the agonist of Tie-2, expressed by the endothelial cells. Ang-2 expressed by endothelial cells classically antagonizes Tie-2 signaling [18]. It promotes vascular leakage [19] and is described as the pro-inflammatory angiogenic factor [20, 21], mostly studied in tumor vasculature. Ang-2 can also act as a context-dependent Tie-2 agonist [22, 23]. Thus, Ang-2 is known as a 'multifaceted cytokine' involved in angiogenesis as well as inflammation [24]. Adding to its ambiguity, Ang-2 has been considered as important part of the vascular niche involved in liver regeneration [25]. A dynamic regulation of Ang-2 by liver sinusoidal endothelial cells mediates hepatocyte proliferation by a complex cross-talk via TGF β -1, thus highlighting its capacity in regenerative angiogenesis [26]. Tie-1 remains largely elusive in this system, though a co-operative role of Tie-1 in Ang-1/Tie-2 signaling ranging to a prominent role in vascular homeostasis has been indicated [27, 28].

Elevated levels of serum VEGF, Ang-2 and soluble Tie-2 have been associated with T2D and vascular dysfunction [29, 30]. Hyperglycemia and free fatty acids ablate Ang-1 mediated Tie-2 signaling in HUVECs [31] whereas Ang-1 protects islets from cytokine induced apoptosis also in absence of Tie-2 and improves islet revascularization post-transplantation [32]. In contrast, Ang-2 has been associated with vascular defects under hyperglycemia [33]. While VEGF-A overexpression increased vascularization near the islet cells and massively altered β -cell function, proliferation and mass, β -cell specific overexpression of Ang-1 or Ang-2 only slightly impaired insulin secretion and glucose tolerance together with marginal altered vascularization, islet mass and morphology [16]. The influence of diabetogenic conditions was not tested in these studies.

The question still remains, whether diabetes progression is a result from altered islet vascularization and whether an increased vascularization improves islet survival or

rather promotes β -cell loss through inflammatory signals brought by the vessels. Therefore, in this study, we set to investigate the changes in islet vessels under diabetogenic conditions together with the function of Ang-2 in islet angiogenesis in the high fat diet induced diabetes mouse model.

Research design and methods

Animals

RIP-rtTA^{tg/tg} (C57BL/6/CBA background) and Tet-O-Ang-2^{tg/wt} (CD-1 background, backcrossed with C57BL/6) mice were used to obtain β -cell specific Ang-2 overexpressing RIP-rtTA^{tg/tg}; Tet-O-Ang-2^{tg/wt} mice (kindly provided by A. Powers, Nashville, TN). Briefly, we used the inducible “tet-on” (tetracycline regulated) transgenic system to allow expression of Ang-2 under the rat insulin promoter (RIP) by doxycycline (Dox; a tetracycline derivative). Male RIP-rtTA; Tet-O-Ang-2 and RIP-rtTA littermate control mice between 8 to 10 weeks of age were given a normal chow diet (ND) (Harlan Teklad Rodent Diet 8604 containing 12.2, 57.6, and 30.2% calories from fat, carbohydrate, and protein, respectively; Harlan Teklad, Madison, WI) or a high-fat high-sucrose diet (HFD) (“Surwit,” containing 58, 26, and 16% calories from fat, carbohydrate, and protein, respectively; Research Diets, Inc., New Brunswick, NJ) for 8, 16 or 24 weeks followed by islet isolation. The mice were provided with 1 mg/ml doxycycline in drinking water throughout the experiment with changes of water every 2 days. Male and female C57BL/6, 8-12 weeks of age were used for islet isolation and culture. All animals were approved by the Bremen Senate in agreement with the §8 of the German animal protection law.

Metabolic tests

Intraperitoneal glucose tolerance test (IPGTT): Mice were fasted for 14-16 hours overnight and injected intraperitoneally with 1g/kg body weight glucose (40%; B. Braun, Melsungen, Germany) and blood glucose levels were measured at 0, 15, 30, 60, 90, and 120 minutes post injection.

Intraperitoneal insulin tolerance test (IPITT): Mice were fasted for 4 hours and intraperitoneally injected with 0.75 IU/kg body weight of recombinant human insulin (InsHuman Rapid, Aventis, Germany) and blood glucose was measured 0, 15, 30, 60 and 90 minutes post injection.

Glucose-stimulated insulin release: Glucose stimulated insulin secretion test was performed on mice after 12-14 hours overnight fasting and intraperitoneal injection of 2 g/kg BW glucose. Blood samples obtained from the retrobulbar plexus at 0 and 30 minutes post injection and the serum was used to measure insulin concentration (Mouse ultrasensitive insulin ELISA, ALPCO Diagnostics, Salem, NH).

Islet isolation and cell culture and treatment

Mouse islets were isolated as previously described [34]. Briefly, islets were isolated by 2 mg/ml liberase (Roche, Mannheim, Germany) injection into the pancreas and digested for 10 minutes at 37 °C in a horizontal shaker. Islet were partly purified by using a density gradient of Histopaque solution (1:1; 1077 and 1119, Sigma) and further purified by hand-picking under a microscope in sterile conditions. Mouse and human islets were cultured in extracellular matrix (ECM-) coated dishes (Novamed Ltd., Jerusalem, Israel)[35] for 48 h through all experiments. For Ang-2 overexpression, islets from RIP-rtTA;Tet-O-Ang-2 and RIP-rtTA control mice were maintained in 10 ug/ml doxycycline prior to and during treatments. Islets were treated with diabetogenic conditions of glucolipotoxicity (22.2mM glucose+0.5mM palmitate) or a cytokine milieu (2 ng/ml IL-1 β , 1000U/ml IFN- γ and TNF- α) for 72 h. MS-1 (ATCC CRL-2279), islet endothelial cell line (kindly provided by P-O Carlsson, Uppsala, Sweden) was cultured in DMEM 5.5mM glucose/5% FCS media and treated with glucolipotoxic (22.2mM glucose+0.25 mM palmitate) or a cytokine milieu (2 ng/ml IL-1 β , 1000U/ml IFN- γ and TNF- α) for 24 h. 100nM Tie-2 kinase inhibitor (Calbiochem, MerckMillipore) was added in parallel to treatments.

Transfection and infection

Ang-2 or Tie-2 downregulation was achieved by using siRNA (ON-TARGETplus siRNA, Dharmacon) in dispersed islets. Transfection was carried out as previously described [36]. Briefly, islets were dispersed into smaller cell aggregates to increase transfection efficiency with accutase for 5-10 minutes at 37 °C and then plated in ECM-coated dishes. 100 pM siScr or siRNA was delivered with Lipofectamine 2000 (Invitrogen). Ang-2 overexpression was obtained with control Adenovirus-GFP or Adenovirus-Ang-2 (kindly provided by H. Augustin, Heidelberg, Germany) 50 MOI for 4 h.

Glucose stimulated insulin secretion of isolated islets

Mouse or human islets cultured in ECM dishes were stimulated by 2.8 mM glucose for 1h followed by 16.7 mM glucose for 1h at 37°C. The supernatants were then used to measure insulin or stored at -20°C. Insulin content was extracted with 0.18 N HCl in 70% ethanol at 4 °C overnight or lysis buffer and determined by a human or mouse insulin ELISA kit (ALPCO).

Immunocyto- and histo-chemical analysis

Isolated islets were fixed with 4% PFA/30 min. Mouse pancreases were isolated and fixed with 4% PFA for 8h at 4°C and then paraffin embedded and cut into 4µm sections. The slides were deparaffinized and immunostaining was carried out post heat antigen-retrieval. The dishes or slides were incubated with the primary antibodies; vessel marker - CD31 (ab28364, Abcam), Ang-2 (F-18, Santa Cruz biotechnology, Inc.) proliferation -Ki67 (mouse-DAKO, human- Invitrogen), insulin (DAKO) or apoptosis - TUNEL-AP kit for dishes or TUNEL-Rx mixture for slides (Roche) followed by secondary antibodies; anti-rabbit, -rat, -goat Cy3 antibodies or anti-guinea pig FITC or biotin (Jackson Immunosearch).

For morphometric analysis, ten sections (spanning the width of the pancreas) per mouse were analysed as described before [37]. Pancreatic tissue area and insulin-positive area were determined by computer-assisted measurements using a Nikon MEA53200 (Nikon GmbH, Dusseldorf, Germany) microscope and images were acquired using NIS-Elements software (Nikon). Mean percent β-cell fraction per pancreas was calculated as the ratio of insulin-positive and whole pancreatic tissue area. β-cell mass was obtained by multiplying the beta cell fraction by the weight of the pancreas. For quantification of blood vessels we measured islet and vessel area by a Region of interest (ROI) tool using NIS-Elements software (Nikon).

Gene expression analysis

Total RNA was isolated from islets or cells with a Trizol extraction system (TriFast-PEQLAB Biotechnology). For quantitative analysis of mRNA we used the Sybr Green real-time PCR kit (Applied biosystems). 18s rRNA or cyclophilin were used as internal housekeeping controls for all experiments and the products quantified by the $\Delta\Delta C_T$ method. Following primers were used: Mouse ANGPT1 Fw 5'-GCCACCATGCTTGAGATAGG-3' and Rev 5'-TTCAAGTCGGGATGTTTGATT-3', ANGPT2 Fw 5'-CGCTGGTGAAGAGTCCAAC-3' and Rev 5'-ATTGTCCGAA-

TCCTTTGTGC-3', Tie-1/TIE Fw 5'-TCCCCCAGATCCTCAGTATG-3' and Rev 5'-ATCTGGCTTGCGAAGTTTCA-3', Tie-2/TEK Fw 5'-CCTTCACCAGGCTGATTGTT-3' and Rev 5'-AATGCATTCCCCGGTATCTT-3', CD31 Fw 5'-TGCTCTCGAAGCCCAGTATT-3' and Rev 5'-TGTGAATGTTGCTGGGTCAT-3', NOS3 Fw 5'-GACCCTCACCGCTACAACAT-3'and Rev 5'- CTGGCCTTCTGCTCATTTTC-3', ICAM-1 Fw 5'- TGGCGGGAAAGTTCCTGTTT-3' and Rev 5'- TAGGAGATGGGTTCCCCCAG-3'.Human ANGPT1 Fw 5'-ATCCCTCCGGTGAATATTGG-3' and Rev 5'-GAATAGGCTCGGTTCCCTTC-3', ANGPT2 Fw 5'-GGCTGGGAAATGAGTTTGTT-3' and Rev 5'-CGGCTGTCCCTGTAAGTCCT-3', Tie-1/TIE Fw 5'-GACTCCGAGATCCAGCTGAC-3' and Rev 5'-CCTGTCCACGTCTATCCACA-3', Tie-2/TEK Fw 5'-TGCTGTCATCAACATCAGCTC-3' and Rev 5'-TGTTGCCAAGCCTCATAGTG-3', CD31 Fw 5'-ATGATGCCCAGTTTGAGGTC-3' and Rev 5'-ACGTC-TTCAGTGGGGTTGTC-3', NOS3 Fw 5'- ACCCTCACCGCTACAACATC -3'and Rev 5'- GCTCATTCTCCAGGTGCTTC -3', ICAM-1 Fw 5'-AACTGGACGTGGCCAGAAAA -3' and Rev 5'- ACAGAGGTAGGTGCCCTCAA -3'.

Western blot analysis

Islet and cell protein was extracted using RIPA lysis buffer and 25 ug protein was loaded for western blot analysis. Actin, tubulin, cleaved caspase 3 (CST, Inc.), Ang-2 and ICAM-1 (SCBT, Inc.) were used at a 1:1000 dilution.

Statistical analysis

All values are expressed as the means \pm SEM. The different groups were compared by Students t test. A P value $<$ 0.05 was considered statistically significant.

Results

Islet vessel area increases in T2D

A causative correlation of islet vessel density and diabetes progression has been suggested for years but it was not clearly known whether there are increased or reduced vessels in islets in diabetes. Using autopsy pancreases, we quantified vessel area within the pancreatic islets by CD31/PECAM-1, located on the surface of endothelial cells and various blood cells. In full accordance with the recent study by Brissova *et al.* [9], there was a 1.34-fold ($p=0.055$) increase in vessel to islet area in T2D donors ($n=10$) compared to non-diabetic controls ($n=6$) (Fig.1A,B).The donor

information is provided in suppl.table 1. We found no correlation in vessel density and BMI of non-diabetic vs. diabetic donors (Fig.1C).

We further investigated the molecular basis of such changes in vessel density in a model of diabetes progression in mice fed a high-fat high-sucrose diet up to 24 weeks. Angiogenic profile analysis after 8 weeks of high fat diet feeding, which already affects glucose tolerance [38] showed upregulation in Ang-2 (1.2 fold, $p=0.05$) and reduction of its receptor Tie-2 on endothelial cells compared to normal diet fed mice but all other vessel markers (Tie-1, CD31) remained unchanged (Fig.1D). Ang-1 is constitutively expressed at low levels, mainly by pericytes. In mouse islets, its expression is less detectable and was unchanged (8 weeks) and further downregulated with time of HFD feeding (data not shown) and a tendency in human T2D islets (Fig.1I, $p=0.09$). At 16 weeks of diet, when robust glucose intolerance occurs together with β -cell apoptosis [37, 39], both angiopoietin receptors Tie-1 and Tie-2 were upregulated (1.5- and 1.8-fold, compared to ND control, Fig.1E) and a clear upregulation in vessel markers analysed; Tie-1 (2.2-fold), Tie-2 (2.1-fold) and CD31 (4.2-fold, $p=0.06$) was seen at 24 weeks (Fig.1F) together with an increase in eNOS (2.2 fold, Fig.1G), involved in vessel dilation and ICAM-1 (2.5-fold, Fig.1H), associated with leukocyte infiltration. No significant change in islet insulin mRNA was seen in HFD mice compared to ND (Fig.S1A-C). VEGF-A upregulation was more prominent at 16 weeks (Fig.S1D), as shown previously [17]. Thus, the angiogenic profile adapts with an increase in the angiopoietin receptors Tie-1 and Tie-2 during vessel expansion and hypervascularization [6] during the progression to T2D.

Human islets isolated from non-diabetic ($n=4$) and T2D ($n=3$) also showed tendencies of higher expression of the vessel markers Tie-1 and Tie-2, but with a high variation among the different donors.

Ang/Tie expression in isolated islets correlates with changes in vessel area

We further asked the question whether such increase in Tie-1/2 receptors is relevant for β -cell failure in T2D progression. Isolated mouse and human islets were used to study the interactions between islet endothelial cells and islet function. To maintain their physiological integrity, mouse and human isolated islets were cultured on extracellular matrix coated dishes [40] and treated with a diabetic milieu of high glucose (22.2 mM) and palmitic acid (0.5 mM) to mimic glucolipotoxicity or with a

mixture of cytokines (cyto; IL1- β , IFN- γ and TNF- α). Mouse as well as human islets showed impaired insulin secretion in response to glucose under glucolipotoxicity and cytokines (Fig.2A,D). Though β -cell function was impaired in both conditions, we found a clear reduction in the endothelial cell area only under cytotoxic and not under gluco-lipotoxic conditions in both mouse (90% reduction, $p < 0.05$) and human (72% reduction, $p < 0.005$) islets (Fig.2B,E) by double immuno-labelling for CD31 (endothelial cells) and insulin (β -cells; Fig.2C,F). The persistent vessels under elevated glucose concentration may support the higher vessel density observation in T2D, while cytokine treatment seems to be deleterious for both β - and endothelial cells.

In sync to the differences in vessel area under both diabetic conditions we also saw an opposing trend in angiogenic expression when RNA analysis of Angiopoietin/Tie angiogenic factors and CD31 was performed in the islets. CD31 expression was upregulated under gluc/palm in human islets (2.2-fold) and downregulated under cytokine treatment in both mouse (20% reduction, $p < 0.05$) and human (47% reduction, $p < 0.05$) (Fig. 2G,J).

Ang-1, the agonist for the Tie-2, was downregulated under high glucose in human and upregulated under cytokine treatment in mouse (6.2-fold, $p < 0.05$) with only a tendency in human islets. In contrast, Ang-2, the classical antagonist, was upregulated under high glucose (3.2-fold in human, $p < 0.05$) and down under cytokines in both mouse (34% reduction, $p < 0.005$) and human (56% reduction, $p < 0.005$; Fig.2H,K,M).

We found no significant differences in Tie-1 expression under high glucose/palmitate in mouse but reduction in human islets under cytokine treatment. Tie-2 on the other hand was down-regulated under high glucose and cyto in human islets (Fig.2J) whereas it was upregulated under cytokine treatment in mouse islets (Fig.2I). Although there were differences in Tie-1/Tie-2 receptor expression in mouse and human islets, the ligands seem to follow a similar trend, with especially significant Ang-2 upregulation in glucolipotoxicity and downregulation under cytotoxicity- the latter paralleled with the regulation of CD31 .

Ang-2 over-expression basally impairs islet function but protects from cytokine induced apoptosis in isolated islets

Seeing the upregulation of Ang-2 by glucolipotoxicity and its reduction by cytokines in human islets, we were intrigued by the ambiguous role of Ang-2 and its direct or indirect- via endothelial cells, effect on islet function in diabetes.

We initially used a downregulation approach to model the situation under cytokines to see if there is an effect on islet survival or function. Downregulation of Ang-2 using siRNA in human islets had neither an effect on glucose stimulated insulin secretion nor on islet survival, although ICAM was upregulated by Ang-2 silencing in the cytokine condition (Fig.S2A,B).

Ang-2 was then overexpressed to investigate whether antagonistic Tie2 signals promote β -cell function and survival. β -cell specific Ang-2 upregulation was obtained in islets isolated from mice expressing myc-tagged Ang-2 under the rat insulin promoter conditionally via the reverse tetracycline activator (RIP-rtTA;Tet-O-Ang-2)[16] and their culture in presence of doxycycline (Fig.3A). Ang-2 over-expression remarkably impaired islet function at basal conditions (0.5-fold, $p < 0.005$) as indicated by the GSIS. In contrast, Ang-2 overexpression in cytokine treated mouse islets, where GSIS was impaired, had no additive effect (Fig.3B).

Ang-2 not only has a dual role on Tie-2 signaling with antagonistic as well as agonistic effects, but also interacts with other receptors like the integrins[21, 22]. By using a Tie-2 kinase inhibitor we and found that Tie-2 inhibition modulates the effects of Ang-2 overexpression with reduction in β -cell function at basal level and no effect under cytokine treatment.

We also investigated the effect of Ang2/Tie2 on β -cell survival by analyzing apoptosis by the TUNEL-assay (Fig.3C,D) and proliferation by Ki-67 staining (Fig.S2C) in mouse islets. No effect on proliferation but protection from cytokine induced apoptosis was seen with Ang-2 over-expression in mouse islets (41% reduction, $p < 0.05$, rtTA vs Ang2-rtTA (Fig.3D). Western blot analyses from treated human islet lysates revealed a similar protection from cytokine induced apoptosis with Ang-2 overexpression and Tie-2 inhibition, seen by lower cleaved caspase 3 (Fig.3G,H).

Along with effects on survival, Ang-2 overexpression and Tie-2 inhibition reduced the expression of endothelial inflammatory marker ICAM-1 in mouse (Fig.3F) and human

islets (Fig.3G,H). The effect of Tie-2 inhibition was reproducible by downregulation of Tie-2 using siRNA in human islets (Fig.S2B) and in the mouse endothelial cell line MS-1 (Fig.S2D). Tie-2 inhibition also resulted in reduced caspase 3 in endothelial cells under cytotoxic conditions (Fig.S2E).

We could confirm the protective role of Ang-2 at cytotoxic conditions by the opposite experiments. Downregulation of Ang-2 exacerbated inflammation, seen by tendency of ICAM-1 upregulation in MS-1 cells (Fig.S2F) and human islets under cytotoxic conditions (Fig.S2B).

Thus, inhibition of Tie-2 signaling at basal conditions impairs insulin secretion. In contrast, when vessels are lost and Ang-2 expression is pathologically reduced by cytokine treatment, its overexpression protects from cytokine induced apoptosis. Further Ang-2 reduction potentiates the deleterious effects of cytokines. This indicates towards a classic antagonistic role of Ang-2 on Tie-2 signaling.

Ang-2 over-expression leads to islet hypovascularization and β -cell failure in response to HFD

Ang-2 was transiently upregulated under HFD and islets were hypervascularized towards progression to T2D from 8 weeks to 24 weeks in mice, as well as under glucolipotoxicity *in vitro*. Thus we set to investigate whether Ang-2 plays a role in causing hypervascularization. Rip-rtTA;tet-O-Ang-2 and the control Rip-rtTA [16] littermates were fed a high-fat high-sucrose diet and Ang-2 expression induced by dox water for 16 weeks during the diet. β -cell-Ang-2 overexpression was confirmed in the Rip-rtTA;tet-O-Ang-2 mice (Fig.S3A). During the 16 weeks feeding, HFD fed mice gained 46% and ND fed mice gained 9.3% weight; there was no effect on weight gain or food intake by Ang-2 overexpression (Fig.S3B,C). HFD feeding lead to impaired glucose and insulin tolerance, compared to ND fed mice. Ang-2 overexpression did not have any significant effect on glucose homeostasis under ND or HFD (Fig.4A,B). HFD-feeding resulted in markedly higher fasted serum insulin in controls ($p < 0.05$) compared to the ND fed mice, sharp attenuation of insulin secretion at 15 and 30 min post i.p. glucose challenge (Fig.4C, $p < 0.05$) and fully abolished glucose stimulated insulin secretion (Fig.4D, $p < 0.005$).

In line with a previous study [16], Ang-2 had a tendency for impairment in insulin secretion under normal diet ($p = 0.06$ vs. ND control), but the HFD mice showed significantly higher insulin at 15 min post glucose injection ($p < 0.05$). This did not

reflect in changes of the stimulatory indices when compared to control ND or HFD mice (Fig.4D). Therefore, it was confirmed that post-natal Ang-2 overexpression over 4 months in the β -cells induces a minor impairment in insulin secretion under normal diet but does not affect glucose homeostasis in response to HFD.

In WT mice HFD feeding resulted in an increased β -cell mass, compared to ND fed mice (Fig.4E). Ang-2 over-expression had no effect at ND, but resulted in a lower β -cell mass in the HFD (Fig.4E; $p < 0.05$).

To address whether this reduction in β -cell mass might be stemming from an effect on β -cell survival, we looked at proliferation and apoptosis, using Ki-67 and TUNEL-assay, respectively. There was no significant difference in cell proliferation with diet or Ang-2 expression (Fig.S3D) but increased β -cell apoptosis by Ang-2 overexpression (Fig.4F, $p < 0.05$).

Under the normal diet, Ang-2 overexpression significantly increased the islet vessel area, with more red blood cells filled vessels, (Fig.4G, $p < 0.05$ vs rtTA ND), but a significant hypervascularization occurred by Ang-2 under the HFD ($p < 0.05$, vs rtTA ND). The vascular changes induced by Ang-2 overexpression were also reflected by mRNA Ang-1 downregulation (Fig.S3E) and upregulation of VEGF-A, Tie-1, Tie-2 and CD31 (Fig.S3F) under normal diet. HFD-induced endothelial inflammatory status worsened with Ang-2 overexpression as seen by induction VCAM-1 and E-Selectin, involved in leukocyte infiltration (Fig.S3H), which may directly lead to increased β -cell apoptosis, although a further functional decline did not occur.

Discussion

In this study we report an increased islet vessel area in T2D, which is in agreement with a very recent study [9]. Such observation was rather surprising, since it has been known for many years, that either a lower islet vessel density or a dysfunctional endothelium is paralleled with diabetes progression.

The importance of a physiological balance of islet vascularization by adaptive angiogenesis for β -cell mass expansion and function was questioned; shown to be negligible on one [7, 8], but to be indispensable on the other hand [41]. Especially the angiogenic factor VEGF-A promotes islet re-vascularization but directly impairs β -cell proliferation leading to a progressive loss in β -cell mass in response to HFD [14, 17]. Also enhanced c-Kit receptor signaling improves islet vasculature and function in

aged mice but elicits an inflammatory response and impairs islet function in response to HFD [42]. VEGF-A [14, 15, 43] and several endothelial derived molecules, collagen IV, thrombospondin-1 [44] and β 1-laminin modulate islet function *in vitro*. Endothelial- β -cell co-culture leads to the formation of pseudoislets [45], and endothelial conditioned medium improves islet function [46]. Ang-1/Tie-2 signaling promotes cell-cell contacts and contact to extracellular matrix [47, 48].

The Ang/Tie angiogenic cascade also participated in the adaptive increase in islet vascularization with Ang-2 upregulation early during high fat/ high sucrose feeding in mice and a persistent Tie receptor upregulation through 24 weeks of the diabetogenic diet, together with upregulated vessel markers eNOS and CD31 at 24 weeks on a high fat diet.

Ang-2 induced vascular defects have been observed under hyperglycemia in chick pancreases transplanted to STZ-treated mice [33]. This implies a role of Ang-2 in inducing vascular anomalies. A resultant compensatory upregulation of Tie-1/-2 to restore vessel stabilization [49] might be in play as seen at 24 weeks of HFD. Ang/Tie were differentially regulated during HFD feeding and we hypothesized a role for Tie signaling driving islet angiogenesis and function in T2D.

Ang-2 overexpression caused islet hypervascularization with a slight impairment in insulin secretion under a normal diet, owing to the vascular defects causing irregular blood perfusion. Despite this minor defect, there was no change in glucose tolerance or insulin sensitivity in Ang-2 overexpressing mice.

On the other hand, in a HFD-induced obesity and insulin resistance model, β -cell specific Ang-2 overexpression led to hypovascularization, reduced β -cell mass with increased β -cell apoptosis. From this data, one can clearly assume, that adaptive hypervascularization is indeed necessary for the maintenance of β -cell survival under situations of higher insulin demand by the β -cell.

Hypervascularization in T2D was supported by our findings in human autopsies and mouse islets from HFD fed mice as well as *in vitro* studies in mouse and human islets. Significantly upregulated levels of Ang-2 under glucolipotoxicity occurred along with a persistent vessel area despite complete ablation of β -cell function.

Impairment in Tie-2 receptor signaling in hyperglycemia and elevated fatty acids via Ang-1 downregulation has been reported [31]. Thus, the inverse regulation of Ang-1/Tie-2 and Ang-2 in glucolipotoxicity points to a rather beneficial role of Tie-2

signaling in islets. Glucolipototoxicity in β -cell failure is classically known [50, 51] but the endothelial cell number is reduced in parallel to β -cell dysfunction in the culture conditions we used.

While glucolipototoxicity induced CD31 expression together with an increase in Ang-2 in human islets, pro-inflammatory cytokines had a drastic effect on the endothelial cells; with loss in CD31 and Ang-2 downregulation. We did not observe any effect on β -cell survival upon modulating Ang-2 or Tie-2 in cultured islets under glucolipototoxicity. But at the conditions of reduced islet vessel density reduced Ang-2 at cytokine treatment *in vitro*, Ang-2 overexpression significantly prevented cytokine-induced apoptosis in mouse and human islets, pointing again to the importance of maintaining physiological balance of angiogenic factors.

The protective effect of Ang-2 on cytokine induced apoptosis in islets and re-vascularization has already been shown [32]. Now, this protection was also induced by Ang-2, rather classically known to be pro-inflammatory, e.g. by sensitizing endothelial cells to the effect of TNF α [21], but also shown to be anti-apoptotic in lymphatic endothelial cells [52]. Ang-2 can assert beneficial effects in a Tie-2 independent manner through integrins [22]. In this study, Ang-2 triggered β -cell apoptosis in HFD-induced diabetes, confirming its widely studied role in vascular leakage [19, 20]. This was supported by the VCAM-1 and E-selectin upregulation in islets from Ang-2-overexpressing HFD mice. In contrast, we also show the opposing Ang-2 beneficial effects *in vitro* on apoptosis protection, which were indeed confirmed by inhibiting Tie-2 *in vitro* in human islets and in the mouse endothelial cell line. In contrast to its protective role on apoptosis, *in vitro* β -cell function was impaired by Ang-2 as well as by Tie-2 inhibition, affirming the antagonistic role of Ang-2 in pathophysiological conditions and suggesting that an imbalance in angiogenic factors is deleterious for homeostasis. Thus, a physiological balance of Ang-2 is important for maintaining β -cell survival and function, and may explain its ambiguous effects.

In summary, our study shows that a functional vascular adaptation together with the physiological preservation of Ang-2/ Tie-2 signaling under diabetic conditions is indeed highly important for maintaining β -cell survival and function.

Acknowledgments

We thank Jennifer Bergemann and Katrischa Hennekens (University of Bremen) for technical assistance, Hellmut Augustin (Dkfz-Heidelberg) for the Ang2-adenovirus.

This work was supported by the German Research Foundation (DFG, Heisenberg Program), the European Research Council (ERC) and by German Diabetes Center grant (DZD) from the BMBF. Human islets were provided through the JDRF award 31-2008-413 (ECIT Islet for Basic Research program) and by the integrated islet distribution program (IIDP). Autopsy pancreases were provided by the National Disease Research Interchange NDRI, supported from the NIDDK.

Author contributions

- Designed and performed all experiments, analyzed data and wrote the paper: PS
- Performed experiments, analyzed data: NL
- Performed experiments and discussed results: AA
- Contributed analytic tools: JKC, FrP, ACP, MB
- Contributed analytic tools and discussed data: POC, JO
- Supervised the project, designed experiments and wrote the paper: KM

Conflicts of interest

Authors have no conflict of interest regulations regarding this submission

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Figure legends

Figure 1: Islet vessel density increases in T2DM

(A) Representative images of pancreatic sections from non-diabetic controls (Con) and type diabetic (T2D) donors, immune-labelled for CD31 (red) and insulin (green). (B) Graphs showing ratio of vessel area to islet area, Con (n=6) and T2D (n=10). (C) Plot showing correlation of vessel density to BMI. qPCR analysis of isolated mouse islets from C57BL/6 WT mice kept on normal diet (ND) or high-fat high-sucrose diet (HFD) Ang-2, Tie-1, Tie-2, CD-31 (D) 8 weeks, n=4/group (E) 16 weeks, n=9/group (F) 24 weeks, n=7/group (G) eNOS (H) ICAM-1 at 8, 16 and 24 weeks (I) qPCR analysis of isolated islets from non-diabetic (Con) and type 2 diabetic (T2D) subjects, showing expression of Ang-1, Ang-2, Tie-1 and Tie-2. *p<0.05, **p<0.005, HFD vs ND or T2D vs Con

Figure 2: Ang/Tie expression in isolated islets correlates with changes in vessel area

Isolated WT mouse and human islets were cultured for 3 days in control condition, 11.1 or 5.5mM glucose for mouse (11.1) and human (5.5) or treated with diabetic conditions of 22.2mM glucose + 0.5mM palmitic acid or mixture of cytokines 2 ng/mL IL-1 β , 1000U IFN- γ and TNF- α (cyto). (A,D) Islet insulin secretion is shown by the stimulatory index assessed by 16.7/2.8 mM glucose stimulation, for mouse (n=4) and human islets (n=5) (B,E) Graph shows a ratio of the quantification of vessel area to islet area for mouse and human islets (C,F) The islets were fixed and immune-labelled for vessel (CD-31, red) and islet (insulin, green). qPCR analysis of treated islets for (G-I) Mouse CD-31, Ang-1, Ang-2, Tie-1, Tie-2 (n=4-5) (J-L) Human CD-31, Ang-1, Ang-2, Tie-1, Tie-2 (n=3-4). All genes have been normalized to PPIA or 18s as housekeeping control. *p<0.05, **p<0.005, treated vs 11.1 or 5.5

Figure 3: Ang-2 over-expression impairs islet function but protects from cytokine treatment in isolated islets

Isolated islets from RIP-rtTA;tet-O-Ang-2 and RIP-rtTA mice were cultured for 3 days in presence of 10µg/ml doxycycline for Ang-2 overexpression. Mouse or human islets were cultured in 11.1 or 5.5mM glucose for mouse (11.1) and human (5.5) or treated with diabetic conditions of 22.2mM glucose + 0.5mM palmitic acid or mixture of cytokines 2 ng/mL IL-1β, 1000U IFN-γ and TNF-α (cyto). **(A)** Western blot from treated mouse islets shows myc-Ang-2 **(B)** Stimulatory index is represented by normalisation to control condition (n=4) **(C,D)** Treated mouse islets fixed post-GSIS and apoptotic cells detected by TUNEL-assay. Representative images from different treatments are shown **(E,F)** qPCR data from mouse islets overexpressing Ang-2. **(G,H)** Western blot showing myc-Ang-2, ICAM-1, cleaved caspase 3 and actin/tubulin as housekeeping control, in human islets overexpressing Ang-2 by Ad-Ang-2 or control Ad-GFP (MOI=50) or treated with 100 nM Tie-2 inhibitor for 72 h. *p<0.05, **p<0.005, treated vs 11.1 or 5.5, #p<0.05, cyto+Ang-2 vs cyto

Figure 4: Ang-2 over-expression leads to islet hypervascularisation and β-cell failure in response to HFD

β-cell specific overexpressing male Rip-rtTA;tet-O-Ang-2 (Ang2-rtTA) and Rip-rtTA (rtTA) were kept on a normal diet (ND,n=8 mice) or high-fat high-sucrose diet (HFD,n=19 mice) and Dox water (1mg/ml) for 16 weeks **(A,B)** Blood glucose levels (mg/dL) from intraperitoneal glucose and insulin tolerance tests performed at 16 weeks post HFD **(C,D)** Glucose stimulated insulin secretion, showing levels of serum insulin and stimulatory index **(E)** β-cell mass analyses on pancreatic sections n=5-6 mice/group **(F)** Apoptotic cells labelled by TUNEL-assay, 10,000 β-cells/mouse, n=6 mice/group **(G)** Islet vessel area quantified by CD31/Insulin co-staining, from 100 islets/mouse, n=6 mice/group **(H)** Representative images from CD31 (red)/Insulin (green) co-staining in mouse pancreatic sections. *p<0.05, **p<0.005 vs rtTA ND, #p<0.05 vs rtTA HFD, §p<0.05 vs Ang2-rtTA ND

Table S1

Donor information – age, gender and BMI, of autopsies obtained from non-diabetic and diabetic subjects,

Figure S1

qPCR analysis of isolated mouse islets from C57BL/6 WT mice kept on normal diet (ND) or high-fat high-sucrose diet (HFD), Insulin genes, Ins1, Ins2 at **(A)** 8 weeks,

n=4 **(B)** 16 weeks, n=3 **(C)** 24 weeks, n=4 **(D)** VEGF-A expression at 8, 16 and 24 weeks. All genes were normalized to PPIA

Figure S2

Human islets were transfected with siRNA (siAng-2 or siTie-2) and control siScr. Islets were treated with diabetic conditions of 22.2mM glucose + 0.5mM palmitic acid (GP) or mixture of cytokines 2 ng/mL IL-1 β , 1000U IFN- γ and TNF- α (cyto) for 72 h **(A)** Stimulatory index normalized to control condition, n=3 **(B)** Western blot analyses of human islet lysates showing ICAM-1, cleaved caspase-3 and actin.

Isolated islets from RIP-rtTA;tet-O-Ang-2 and RIP-rtTA mice were cultured for 72 h in presence of 10 μ g/ml doxycycline for Ang-2 overexpression and treated as above **(C)** Treated islets fixed post-GSIS and colabelled with Ki-67/Insulin, Graph showing %Ki-67 positive β -cells, n=3 **(D,E)** Western blot analyses of MS-1 cells, transfected with siRNA (siTie-2) and control siScr or treated with 100nM Tie-2 inhibitor and treated as above for 24 h. Blots show Ang-2, ICAM-1, cleaved caspase-3 and tubulin or actin **(F)** qPCR data for Ang-2, CD31 and ICAM-1 in MS-1 cells transfected with siAng-2 or control siScr and treated as above for 24 h, n=5, **p<0.005, *p<0.05 vs siScr 5.5

Figure S3

β -cell specific overexpressing male Rip-rtTA;tet-O-Ang2 and Rip-rtTA were kept on a normal diet (ND, n=8 mice) or high-fat high-sucrose diet (HFD, n=19 mice) and Dox water (1mg/ml) for 16 weeks **(A)** Representative images from mouse pancreatic sections showing dox induced β -cell specific Ang-2 expression (red) in mouse islets (green) **(B)** %weight gain and **(C)** food intake/mouse/day over 16 weeks, *p<0.05, **p<0.005 vs rtTA ND **(D)** Graph showing %Ki-67 positive β -cells in mouse pancreatic sections, n=6 mice/group **(E-H)** qPCR analyses of islets isolated from rtTA and Ang-2-rtTA, ND and HFD mice at 16 weeks, Ang-2, Ang-1, VEGF-A, Tie-1, -2, CD31, ICAM-1, eNOS, VCAM-1, E-Selectin

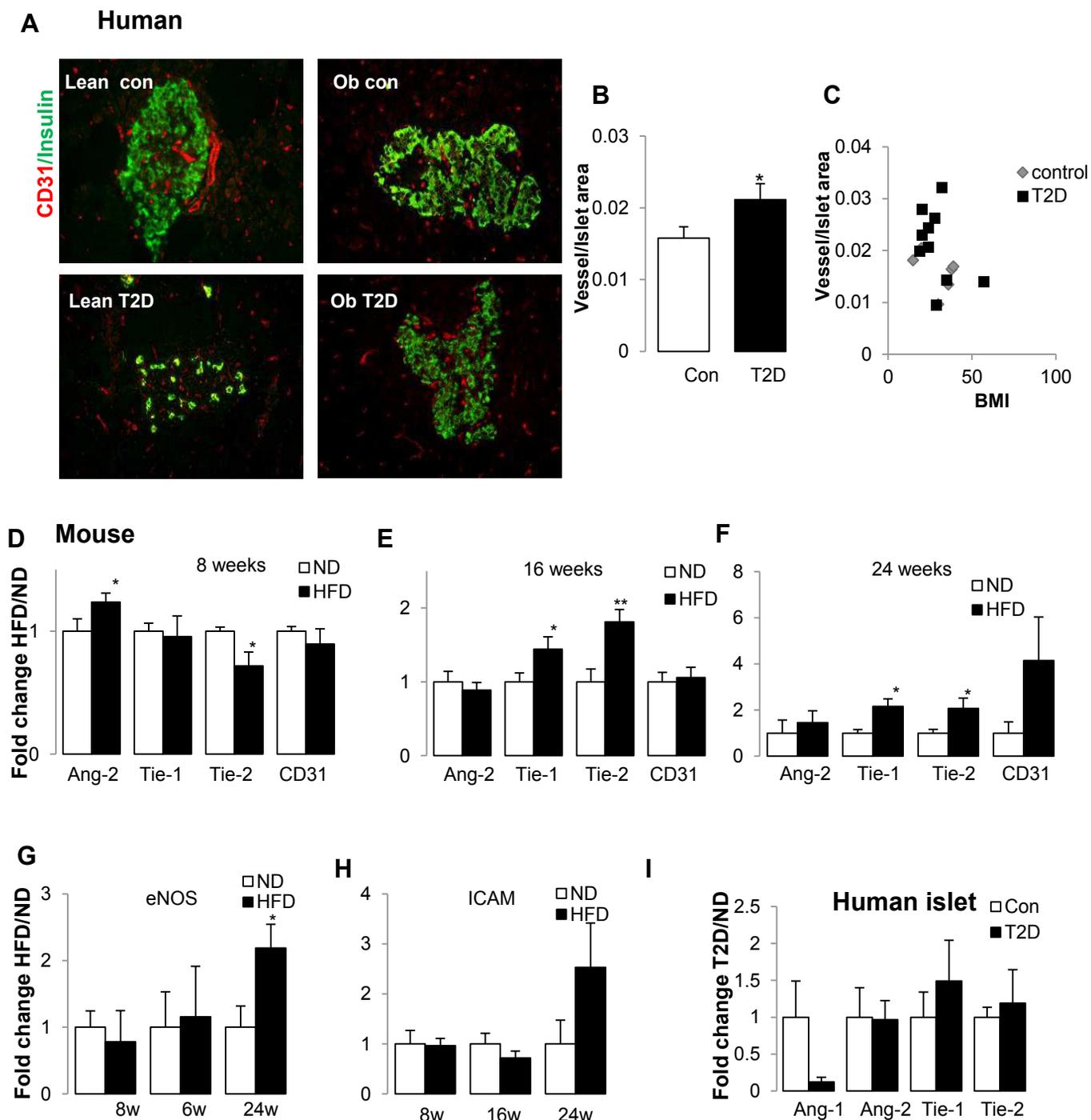


Figure 1

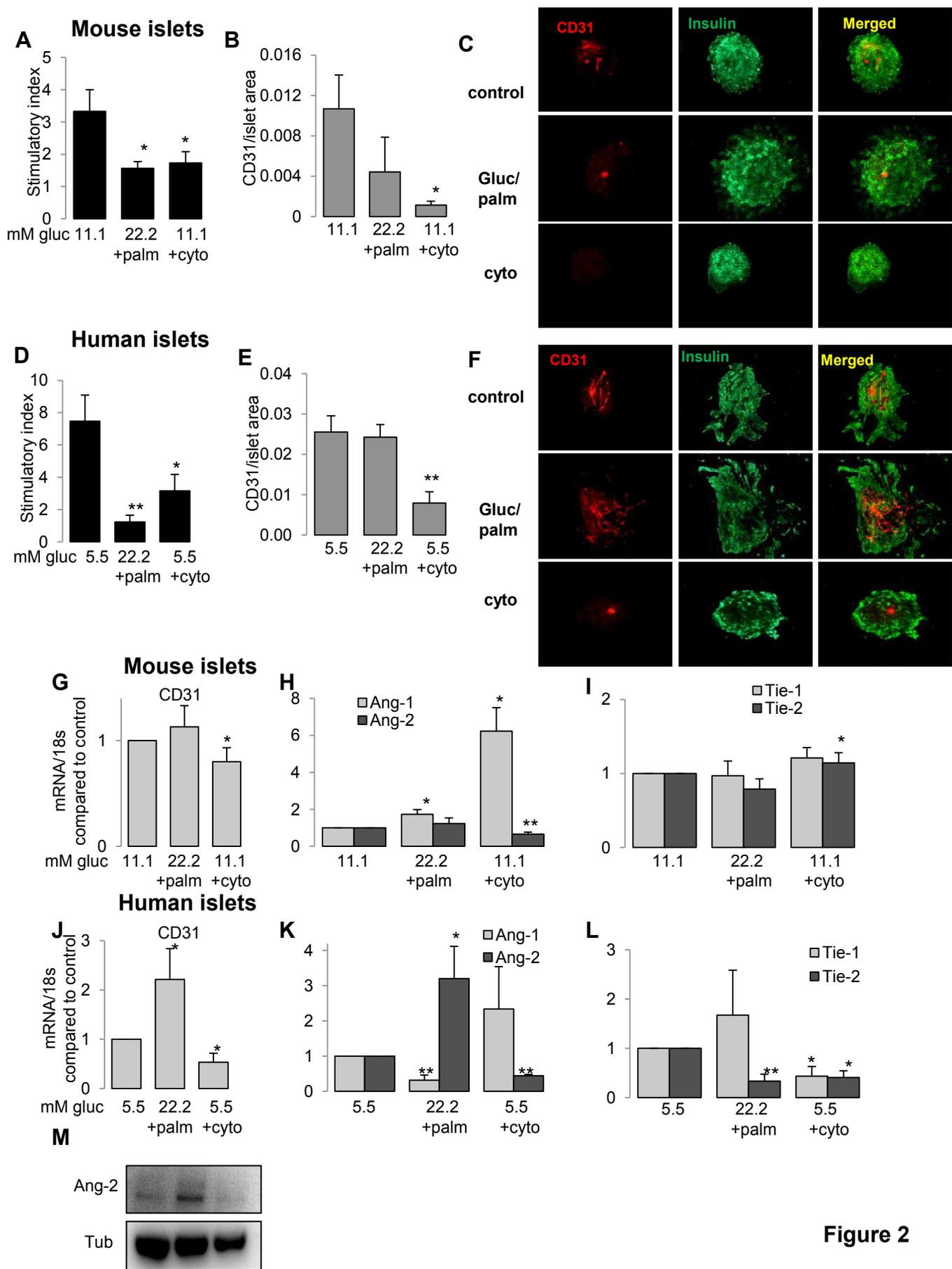
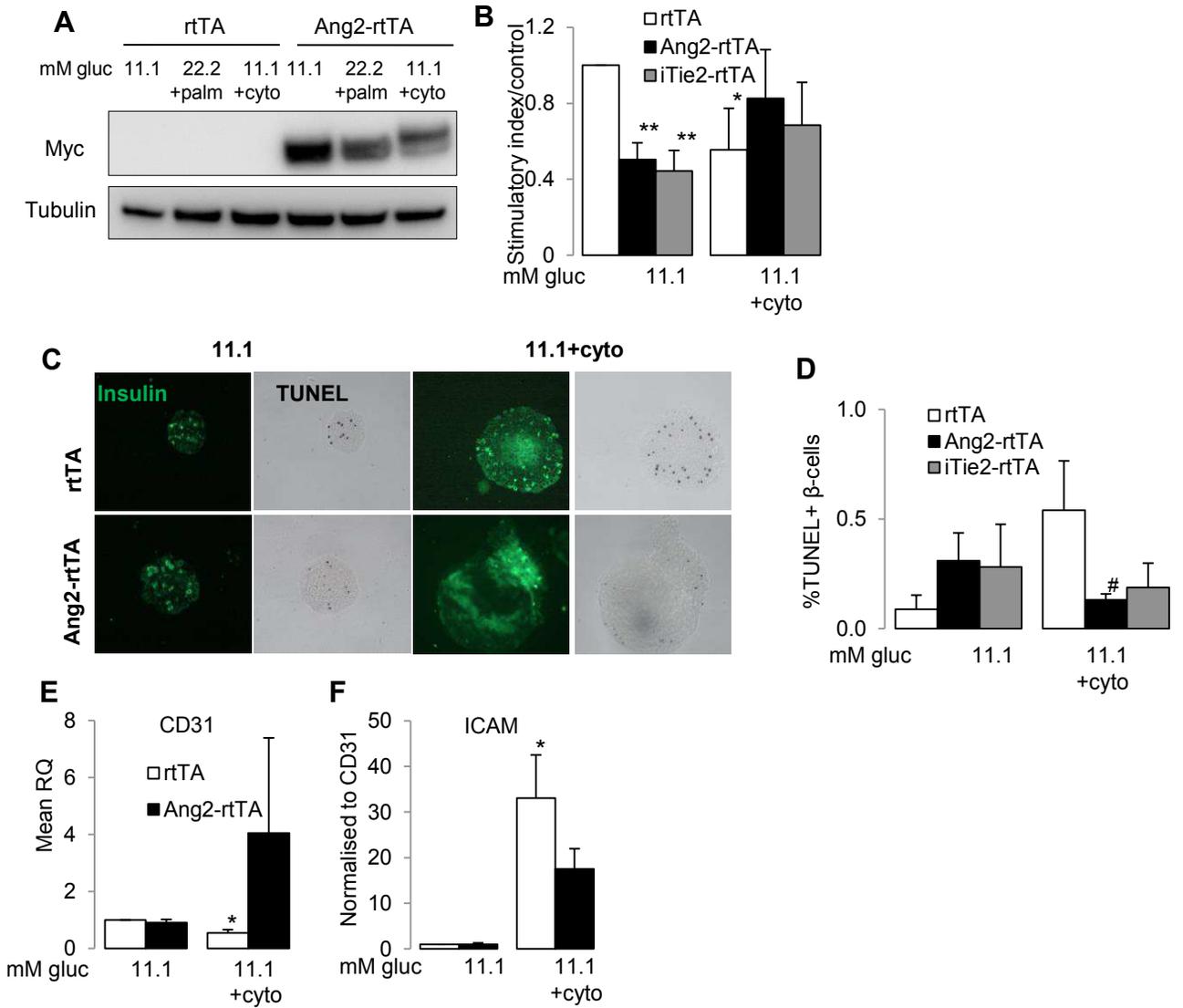


Figure 2

Mouse islets



Human islets

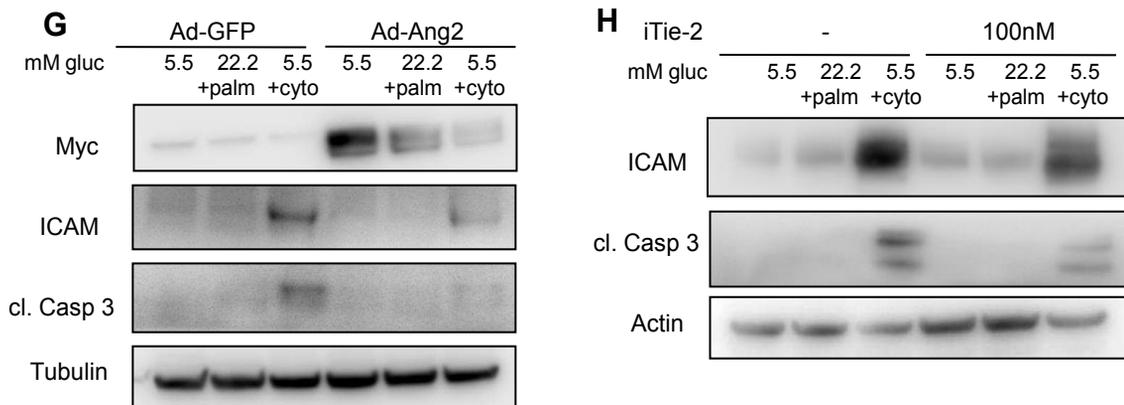


Figure 3

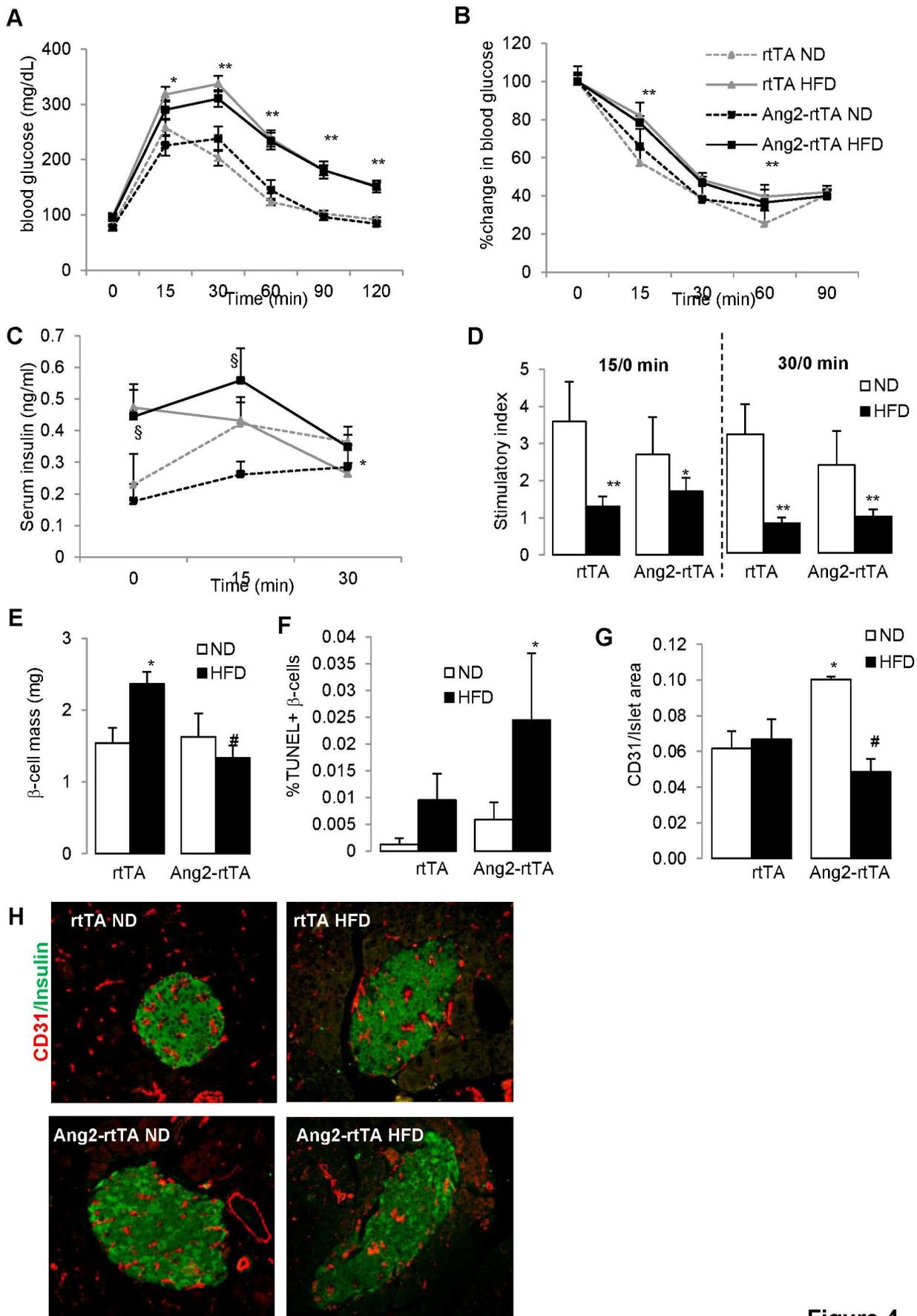


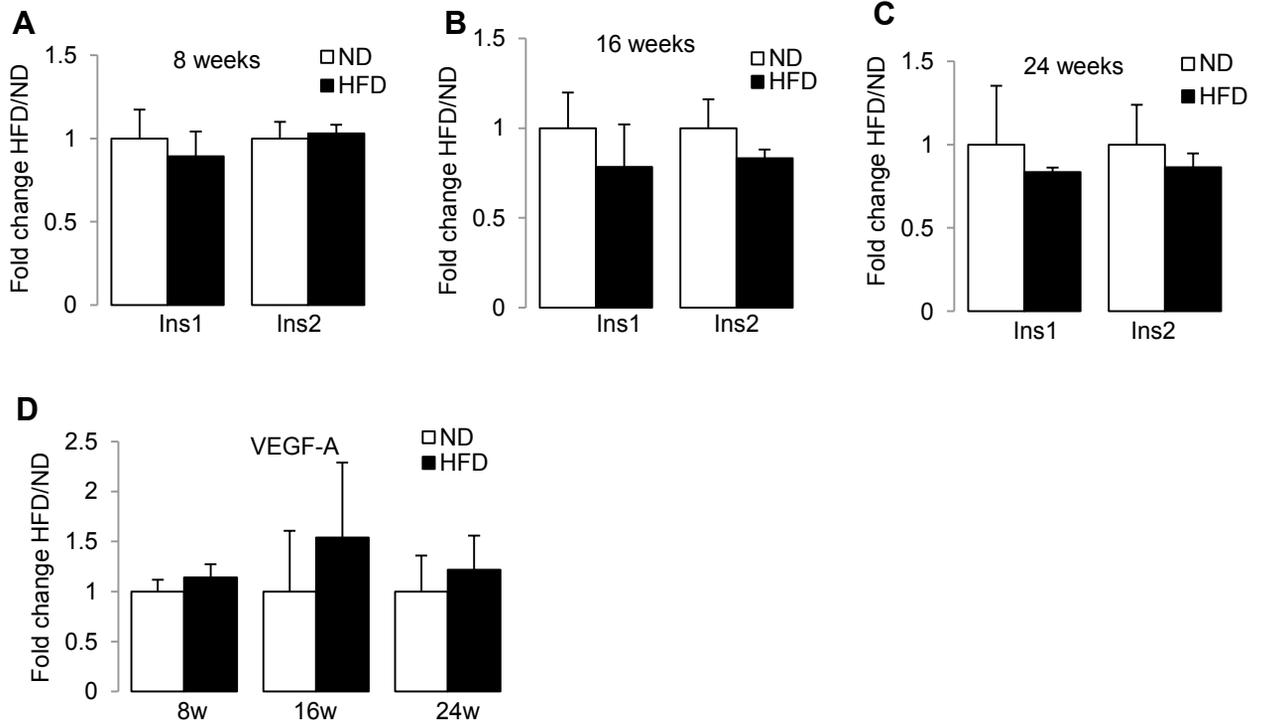
Figure 4

Supplementary figures

Supplementary Table 1

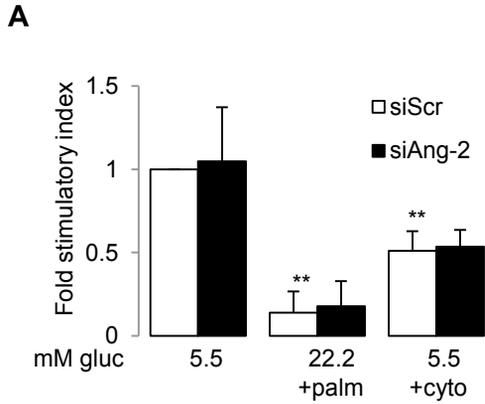
	Non-diabetic (Con)	Type 2 diabetic (T2D)
Age (y)	70.5 ± 4.3	74.1 ± 2.4
Gender (F/M)	2/4	7/3
BMI	29.67 ± 4.1	28.88 ± 3.5

Supplementary figure 1

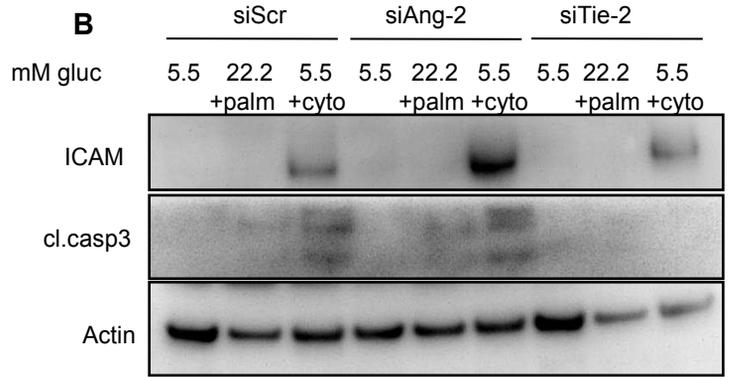


Supplementary figure 2

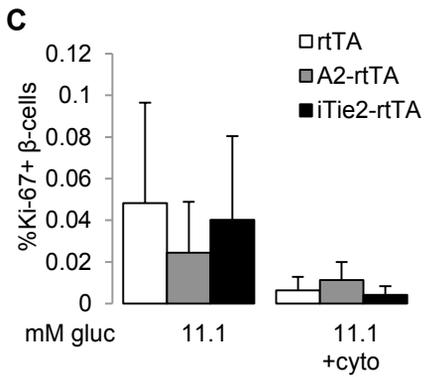
Human islets



B

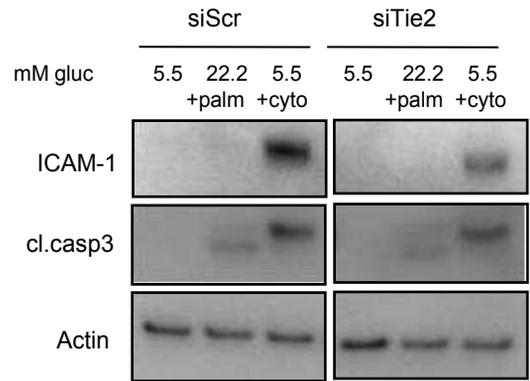


Mouse islets

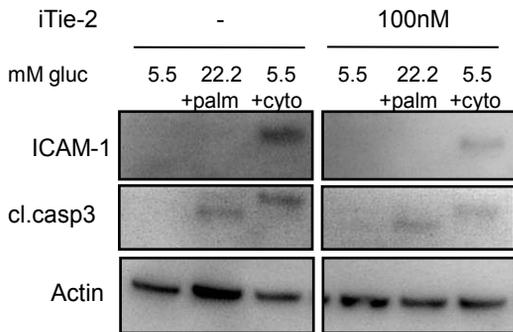


Mouse EC line

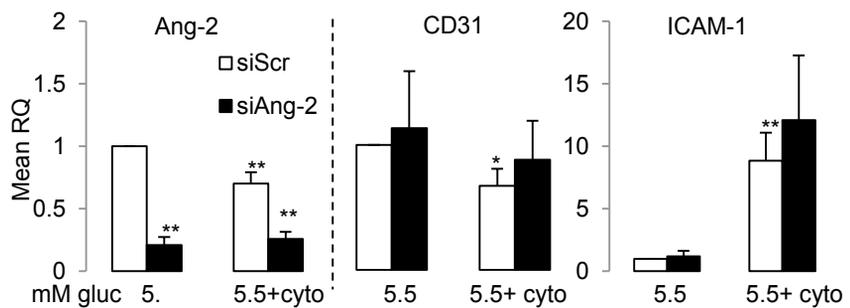
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E

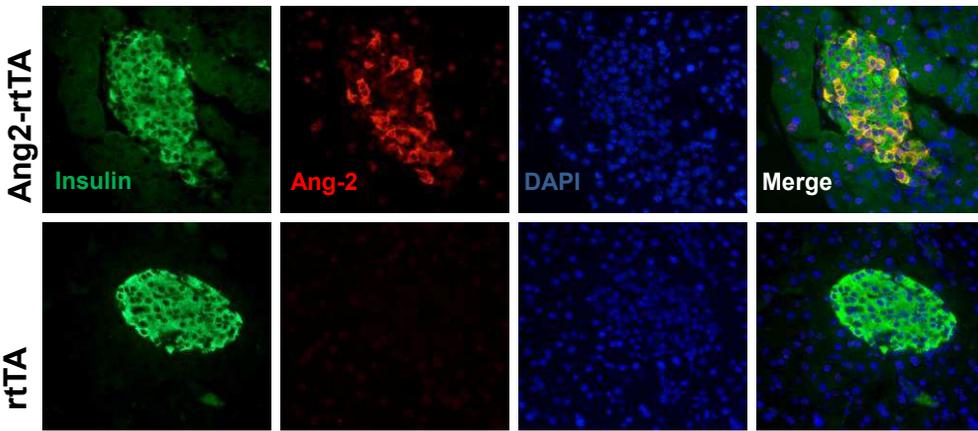


F

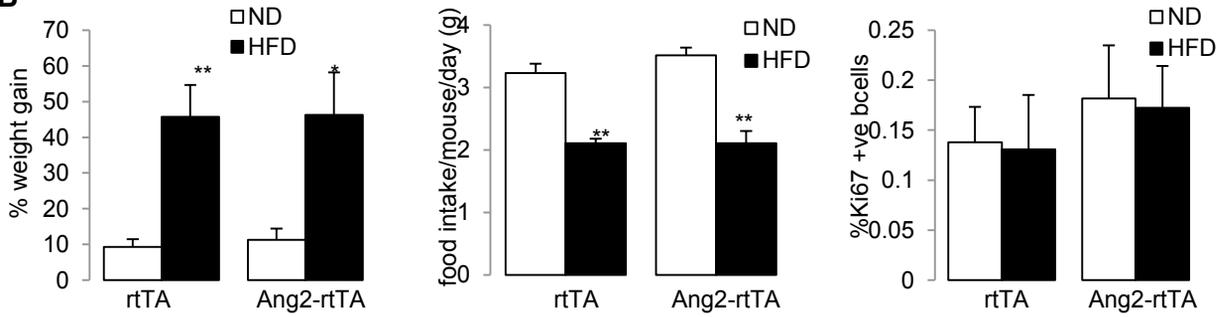


Supplementary figure 3

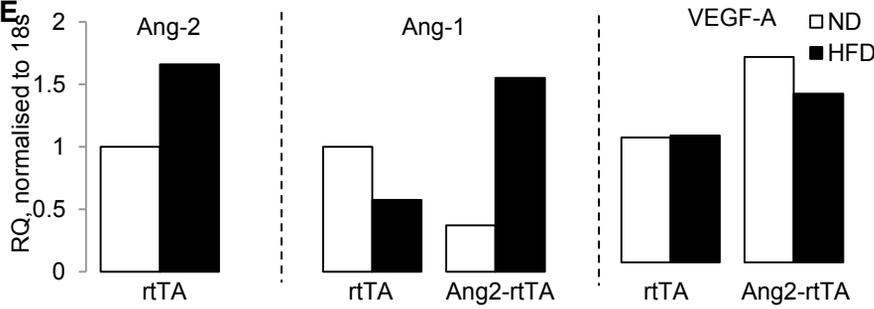
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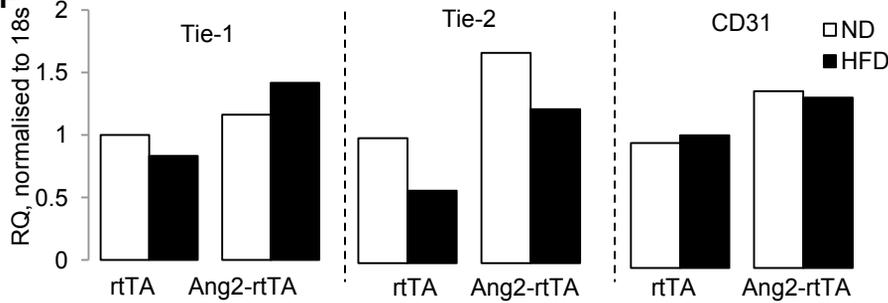
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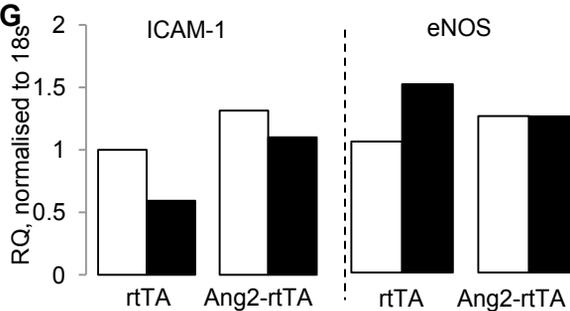
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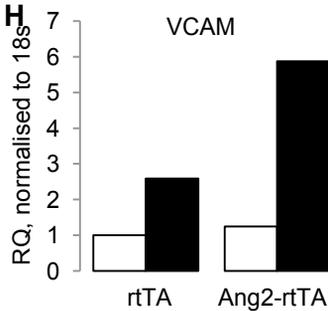
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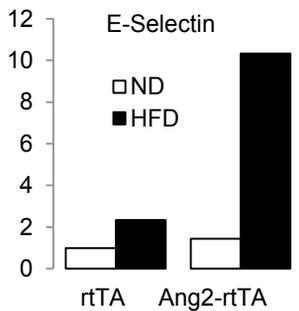
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H



I



C. Discussion and outlook

This doctoral thesis includes three studies carried out on different aspects of diabetes though the focus remains on islet vascularisation in T2D. In the first published study, we showed the restoration of β -cell function and survival from diabetic treatment, in response to 4 days treatment by the DPP4 inhibitor, linagliptin. Human islet cultured in extracellular matrix coated dishes showed a basal proliferative capacity which was ablated and apoptosis was induced upon treatment with high glucose, palmitate, cytokines and H_2O_2 . The efficacy of linagliptin on lowering blood sugar levels was established in 2010 after the Phase III clinical trial [1]. Sitagliptin was the first DPP4 inhibitor to be approved by the FDA in 2006 [2]. Sitagliptin administration prevented occurrence of overt diabetes in NOD mice [3] and linagliptin delayed the onset of diabetes in NOD mice with reduced immune cell infiltration and preserved β -cell mass temporarily but no effect later on [4]. Since, the discovery of DPP4 inhibitors, there have been studies on the efficacy of the drugs on the β -cell survival, function and mass apart from its established effects on insulin sensitivity [5]. Linagliptin and other DPP4 inhibitors are mostly administered in combination with metformin and pioglitazone and not only as monotherapy [6]. GLP-1 actions are largely mimicked by the DPP4 inhibitor but not many studies show its direct effects on islets unlike the well known beneficial effects of GLP-1 on islets survival and function in mice as well as T2D subjects. In this study, we also showed the stabilization of GLP-1 by increased active GLP-1 in islet supernatants from the treated islets. Islet GLP-1 production is prominent under diabetic conditions due to the increased expression of the pro-hormone convertase PC1/3, responsible for cleavage of glucagon and releasing GLP-1 [7]. Thus, the local GLP-1 provides a protective effect on β -cells under diabetogenic conditions. The GLP-1 receptor expression is decreased under hyperglycemia [8] and here we showed restored levels of the receptor expression in human islets. DPP4 also cleaves SDF1 α (1-68) to its inactive form SDF1 α (3-68), thus restoring its effects upon DPP4 inhibition. We also showed reduction in levels of IL-6 and IL-1 β , which are induced upon cytokine milieu. In summary, we show that linagliptin has a protective effect on β -cell turnover and function under diabetogenic conditions, which is

mediated through stabilized GLP-1 and anti-inflammatory/antioxidative stress pathway.

In the second, unpublished study, we show the inhibition of CXCL10 in protection from HFD induced insulin resistance and β -cell failure. We have previously shown the upregulation of CXCL10 in diabetes and as a major cytokine produced in the islets in T1D and T2D [9]. In this study we found anti-CXCL10 antibody to improve glycemia, insulin secretion, insulin sensitivity and β -cell survival and function in mice fed a high fat/high sucrose diet for 16 weeks. Moreover, we also saw reduced levels of inflammatory gene expression in the peripheral tissues obtained from these mice. MDX-1100, a highly specific CXCL10 neutralizing antibody blocks CXCL10 induced calcium flux and cell migration with an estimated IC_{50} in the low nM range [10]. It specifically inhibits CXCL10 and not CXCL9 or CXCL11, the other 2 ligands of the classical receptor for CXCR3 [10]. TLR4 activation in obesity in response to free fatty acids in adipose tissue, hypothalamic region of the brain as well as pancreatic islets has been shown by many studies, thus linking the metabolic and inflammatory bridge in obesity induced insulin resistance and β -cell failure [11]. We have also shown TLR4 to be a binding partner for CXCL10 and thus TLR4 antagonism serves as an attractive target for improving the metabolic syndrome in the face of inflammation. FFAs induction of TLR4 has received considerable attention as well and thus, in our study we imply the antagonism of TLR4 as closing of a deleterious vicious cycle of FFAs induced inflammatory induction in pancreatic islets as well as peripheral tissues. It has entered the phase II clinical trial for rheumatoid arthritis and was well-tolerated, demonstrated clinical activity and no drug related serious adverse events were reported [12]. Though detected a >10-fold induction of CXCL10 mRNA in isolated islets, immunostaining of the pancreas only revealed in few β -cells positively stained for CXCL10 in islets from HFD mice and expression pattern is very similar to CXCL10 expressing β -cells in patients at recent onset of T1D [13]. This might be due the secretion of the chemokine in the serum rather than accumulation in the cell and thus undetectable by immunohistochemistry, similar to observations for IL-6 and IL-1 β [14, 15]. CXCL10 neutralization lead to reduced expression of IL-6 and IL-1 β probably due to mechanism through a feed-back, which reduces

CXCL10 induced signalling. Only in the liver, IL-1 β production failed to decrease, pointing to a CXCL10 independent effect. In contrast, we did not detect changes in inflammatory cytokine profile in response to HFD feeding in the hypothalamus and thus, also no effect was seen by CXCL10 antagonism. In summary, with the robust effect of the anti-CXCL10 antibody on glycemia, insulin secretion and β -cell survival, we provide here a novel target for the treatment of T2D.

Islet vascularisation in T2D has been the main focus of our studies. The islets comprise of 1-2% of the total pancreatic mass but are provided with 10-15% of the blood supply [39]. Thus, they are in close connection with the endothelial cells and the surrounding extracellular matrix, forming a specialized capillary network serving the tight regulatory role of islets in glucose sensing and homeostasis. In our study we observed Ang-2 mediated islet hypovascularization and apoptosis with reduced β -cell mass in the HFD setting. Thus, the adaptive hypervascularization is indeed necessary for the maintenance of β -cell survival under situations of higher insulin demand by the β -cell. Ang-2 lead to lower vascularization in contrast to the hypervascularization induced by VEGF-A though in both models, there is upregulation of endothelial inflammatory markers [16]. These differences might be due to the residual state of the β -cells in either case, which remain to be impaired with hypervascularization causing impairment to a great extent than hypovascularization. Our finding also supports that hypovascularized islets go on functionally well [17] without exerting overt effects on progression to β -cell failure: despite the increase in β -cell apoptosis which resulted in loss of β -cell mass, we didn't observe a worsened diabetic phenotype despite overexpression of Ang-2 over a prolonged period of 16 weeks. This might be explained by the complex role of Ang-2 in physiology, being necessary for an angiogenic response in some peripheral tissues and not in others. HFD feeding also presents a metabolically complex setting with the inter-play of several organs. It might be useful to assess the circulating levels of Ang-2 in the serum to further explain this effect.

Ang-2 overexpression increased the islet vessel area under normal diet conditions with slightly impaired insulin secretion observed in these mice upon

glucose challenge. Ang-2 is known to induce vessel destabilization and this probably also leads to higher β -cell apoptosis under the HFD feeding in Ang-2 overexpressing mice.

Though in vitro Ang-2 seems to play even a protective role under a cytokine milieu with reduced apoptosis in both Ang-2 overexpression as well Tie-2 inhibition. Thus the in vivo observation might be more an effect of the physiological hypoxia induced by lower islet vascularisation associated with necrotic cell death. In the current study we report an increased islet vessel area in T2D, which is in agreement with a very recent study [18]. In presence of VEGF-A, Ang-2 induces increased capillary diameter, remodeling of the basal lamina and proliferation and migration of endothelial cells [19]. On the other hand exogenous Ang-2 induces endothelial death and vessel regression in absence of VEGF-A, which might be the explanation for Ang-2 induced vessel regression observed upon HFD feeding. Systemic Ad-Ang-2 even inhibits tumor growth by tumor microvessel regression in a mouse tumor model [20]. This is a possible link to Ang-2 being associated with lower vessels in diabetes. Anti-angiogenic therapies have been a central dogma of tumor treatments for ages [21]. Ang-2 has been associated with a growing mass of cells and vascularizing the tissue. This cannot be so easily mirrored with the expanding β -cell mass per se since the etiologies of the diseases are completely different. β -cell mass has a tremendous plasticity compared to an ever growing tumor and while expanding β -cell mass is a physiological response to increased insulin demand, it does not necessarily lead to diabetes. Therefore, islet blood flow regulation rather than robust angiogenesis seems to play a central role in β -cell mass expansion. On the other hand a prolonged proliferation pressure on β -cells may exert the consequent hypervascularization observed in T2D and hypoxia may be the cause for inducing vascularization rather than a consequence. This effect is probably inhibited by Ang-2 overexpression and prevents β -cell mass expansion as we observed by lower β -cell mass in Ang-2 expressing HFD mice compared to the control HFD. Overall, Ang-2 prevents β -cell mass expansion and causes islet vessel regression in progression to T2D. Short term effects of Ang-2 overexpression along with HFD feeding might lead to

interesting results clarifying whether there are any effects of Ang-2 during the first phase of insulin demand compensation.

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D. Appendix

1. CXCL10 antagonism improves insulin sensitivity, β -cell function and mass

Payal Shah, Jennifer Bergemann, Nadine Stappenbeck, Pia C. Janssen, Laurianne Giovannoni, Thierry Berney, Pina Cardarelli and Kathrin Maedler

2. DPP-4 inhibitor Linagliptin: effect on β -cell survival and function

Payal Shah,* Amin Ardestani,* Gitanjali Dharmadhikari,* Svenja Laue, Desiree M. Schumann, Julie Kerr-Conte, Francois Pattou, Thomas Klein, and Kathrin Maedler

*Authors contributed equally

CXCL10 antagonism improves glucose tolerance, insulin sensitivity, beta-cell function and mass

Manuscript under revision, submitted to Diabetologia

My contribution: Performed RT-PCR and analysed data

CXCL10 antagonism improves glucose tolerance, insulin sensitivity, β -cell function and mass.

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Running Title

CXCL10 antagonism promotes glycemic control

Keywords

CXCL10; islets; inflammation; diabetes

word count: Abstract: 249, main text: 3.510, figure legends: 580

Abstract

Aims/hypothesis. Inflammation participates in the pathophysiology of both type 1 (T1D) and type 2 diabetes (T2D) and leads to β -cell failure and impaired function. The pro-inflammatory chemokine CXCL10 is expressed in islets in T1D and T2D and leads to β -cell apoptosis and impaired function mediated through TLR4 signaling. In the present study we investigated whether CXCL10 antagonism improves glycemia, β -cell function and survival.

Methods. C57BL/6J mice were fed a high fat/high sucrose diet (HFD) for 16 weeks and treated with an antibody to CXCL10 twice weekly. Glycemia was monitored and pancreas, liver, adipose tissue and hypothalamus were isolated and analyzed by IHC and RT-PCR after 16 weeks.

Results. Anti-mouse CXCL10 antibody prevented diabetes progression; it improved glucose tolerance, insulin sensitivity and completely restored glucose stimulated insulin secretion in the HFD fed mice.

While anti-CXCL10 treated HFD mice showed increased β -cell mass compared to normal diet (ND) controls, β -cells were unable to compensate for the higher insulin demand in response to the HFD in vehicle treated mice. Anti-CXCL10 treated mice showed no β -cell apoptosis under the HFD treatment; CXCL10 positive β -cells were found in HFD but not in ND or anti-CXCL10/HFD treated mice. IL-1 β , IL-6 and CXCL10 mRNA in isolated islets were highly up-regulated in HFD treated mice, which was inhibited by anti-CXCL10. CXCL10 mRNA was up-regulated in epididymal fat and liver and attenuated by anti-CXCL10.

Conclusions. Our results show that CXCL10 antagonism improved β -cell survival and function and support a potential role for anti-CXCL10 in the treatment of diabetes.

Introduction

Increased apoptosis resulting in decreased β -cell mass together with loss of function of the pancreatic β -cells are hallmarks of both, type 1 and type 2 diabetes (T1D/T2D). Therapies, which restore β -cell function and survival are urgently needed. A subclinical

inflammatory status has been observed in serum from patients with T2D. Pro-inflammatory markers are elevated in insulin sensitive tissues such as muscle, liver adipose tissue and in pancreatic islets [1-6]. An overall impairment in glucose sensitivity in these tissues, stemming from and leading to both β -cell failure, indicates an inflammatory crosstalk involved in potentiating the disease progression. β -cells express high levels of pattern recognition receptors (PRR) and cytokine receptors, e.g. TLR2 and 4 [7], IL-1R1 [8] and IL-6R/GPR30 [9]. In contrast to such high expression of PRRs, β -cells express lower levels of anti-oxidative enzymes [10], rendering them sensitive to oxidative stress.

Hyperlipidemia alone or in concert with hyperglycemia, so called “lipoglucotoxicity” can induce such pro-inflammatory state, shown in fat, where elevated free fatty acids lead to impaired insulin sensitivity, as well as in pancreatic islets, where prolonged lipoglucotoxicity initiates a vicious cycle in β -cell destruction [11], both mediated by TLR4 signaling [12, 13]. Consequently, downstream of TLR4, the constant activation of sub-clinical levels of pro-inflammatory cytokines can lead to a high local expression of markers involved in the acute immune response such as IL-1 β , TNF α , IL-8, MCP-1 [14] and to disease development through activation of the classical apoptotic machinery [15]. Recent clinical trials showed that the neutralization of one single cytokine, IL-1 β , a well-known agent for its effects on β -cell dysfunction and destruction when exposed chronically in high levels, normalizes glycemia and reduces inflammation in patients with T2D [16-20].

CXCL10 was observed to play a causative role in diabetes development [21-23]. Increased CXCL10 serum levels are present in both T1D [24, 25] and T2D patients [26]

as well as in patients with high risk to develop T1D [24] and T2D [27]. It acts as a potent chemo-attractant for mononuclear cells, T-cells, B-cells and NK-cells. CXCL10 is present prior to the onset or at least at an early stage of the disease development, observed in the NOD mouse in the pancreas before signs of insulinitis [28, 29]. CXCL10 is also expressed in β -cells in T1D [30] and T2D [5].

Exposure of islets to CXCL10 *in vitro* at similar concentration as produced from diabetic islets leads to apoptosis and impairs β -cell function, this is mediated through TLR4 signals. Pre-incubation with a neutralizing antibody to CXCL10 prevents from CXCL10 induced apoptosis, confirming a CXCL10 specific effect [5]. Similarly, CXCL10 induces apoptosis in hepatocytes during liver injury, which is also mediated through TLR4 [31] and also causes apoptosis in the exocrine pancreas [32] as a possible mechanism for chronic pancreatitis. Also, an association of CXCL10 with both BMI and waist circumference [33] supports an interplay of FFAs with cytokines and chemokines and links obesity, inflammation and diabetes. CXCL10 is present at the onset of T2D in adults [27]. In young schoolchildren, serum levels of CXCL10, IL-6 and IL-18 correlate with obesity and insulin resistance [33] suggesting that CXCL10 may be an important factor in the development of insulin resistance and T2D in youth.

These studies suggest that a pro-inflammatory milieu potentiates the complex pathological disturbances in metabolic diseases. We tested therefore, whether blocking CXCL10 signals using a neutralizing antibody to CXCL10 can restore normoglycemia in an animal model of diabetes, the high fat/ high sucrose fed mouse. Furthermore, we wanted to investigate whether blocking of CXCL10 can prevent the potentiation in cytokine production from insulin sensitive tissue and pancreatic islets.

METHODS

CXCL10 antibodies. Two anti-CXCL10 were used in this study, anti-human CXCL10 (MDX-1100, also known as BMS-936557) and anti-human CXCL10 that crossreacts with mouse species (referred to as anti-mouse CXCL10). Both antibodies and the corresponding isotype control antibodies were made as fully human monoclonal antibody in HuMab® or KM® transgenic mice and re-expressed as IgG1 (kappa) by recombinant DNA technology at Biologics Discovery California (BDC) of BMS (formally named Medarex). The mouse cross-reactive antibody was re-expressed as a mouse IgG1 to reduce the potential immunogenicity of the mAb in the mouse *in vivo* studies.

Animals. Beginning at 6 weeks of age, C57BL/6J male mice originally obtained from The Jackson Laboratory (Bar Harbor, Maine) were fed either a normal diet (ND, Altromin GmbH & Co. KG #1320, containing 11, 65 and 24% calories from fat, carbohydrate and protein, respectively) or a high fat/high sucrose diet (HFD, “Surwit” Research Diets, New Brunswick, NJ, containing 58, 26 and 16% calories from fat, carbohydrate and protein, respectively [34] for 12-16 weeks. Mice were treated twice-weekly with intraperitoneal (i.p.) injections of 10 mg/kg body weight mouse MDX1100 or isotype control (mouse IgG1). Body weight and food consumption were monitored weekly.

The following treatment groups were examined: (1) ND IgG (mice on normal diet and isotype control), (2) HFD IgG (mice on high fat/high sucrose diet and isotype control), (3) ND anti-CXCL10 (mice on normal diet and anti-mouse CXCL10), (4) HFD anti-CXCL10 (mice on high fat/ high sucrose and anti-mouse CXCL10) in 3 independent

experiments. All animals were housed in a temperature-controlled room with a 12-h light/dark cycle and were allowed free access to food and water in compliance with the *Guide for the Care and Use of Laboratory Animals*, and the Bremen Senate in agreement with the National Institutes of Health animal care guidelines and §8 of the German animal protection law.

Intra-peritoneal glucose and insulin tolerance tests. After 4, 8, 12 and 16 weeks of diet and treatment all animals underwent *in vivo* assays. Intraperitoneal glucose tolerance tests (ipGTT) and stimulated insulin secretion were performed after 12 h fasting and intraperitoneal insulin tolerance tests (ipITT) after 5h fasting as described before [35]. Stimulation index for insulin production was calculated by dividing the 30 min insulin (stimulated) by the 0 min value (basal).

Homeostatic model assessment- insulin resistance. HOMA-IR was calculated using the method by Odgaard et al [36]: fasting insulin (ng/mL) x fasting glucose (mmol/L) = HOMA-IR.

Histochemical analyses and β -cell mass. Pancreata were weighed, fixed and apoptosis and β -cell mass were analyzed as described previously [37]. CXCL10 analysis was performed through double-staining the sections with goat anti-mouse CXCL-10 antibody (Santa Cruz Biotechnology, Inc.) and guinea pig anti-insulin (DAKO), nuclei visualized with Vectashield Mounting Medium with DAPI (Vector Laboratories).

Islet isolation and culture. Mouse islets from all groups were isolated as described previously [38] and cultured overnight in RPMI 1640 medium containing 11.1 mM glucose (PAA Laboratories GmbH, Cölbe, Germany). Human islets were isolated from four pancreata of healthy organ donors as described previously [39] and cultured in

CMRL-1066 medium [5]. Islets were exposed to 22.2 mM glucose plus 0.5 mM palmitate [40] with or without 10 µg/ml human MDX-1100 or 10 µg/ml human IgG.

Cytokine expression analyses. Total RNA from epididymal fat pads, liver, hypothalamus and pancreatic islets was extracted from mice after 16 weeks of diet and treatment and isolated using peqGOLD TriFast™ (Peqlab) or RNeasy Mini Kit (Qiagen) for the hypothalamus. RT-PCR was performed using Power SYBR Green PCR Master Mix; Applied Biosystems). Primers see supplementary files.

Calcium flux assay. Murine pre-B 300.19 cells overexpressing human CXCR3 were loaded with FLIPR Calcium 4 dye (FLIPR Calcium 4 kit, Molecular Devices, Sunnyvale CA). CXCL10 (Peprotech, Rocky Hill, NJ) at 3 nM was used to stimulate calcium flux. A titration of BMS-936557 from 13 pM to 600 nM was used to inhibit the response. A maximal calcium response was set with CXCL10 minus antibodies. A baseline response was established with buffer stimulation of cells without CXCL10. Calcium fluxes were read on the Flexstation (Molecular Devices, Sunnyvale, CA). Data was graphed and analyzed with GraphPad Prism software, using nonlinear regression and sigmoidal dose-response curves.

Migration assay. Cells were loaded with Bis (acetoxymethyl) 2,2':6',2''-terpyridine-6,6''-dicarboxylate (BADTA) chemiluminescent migration reagent (PerkinElmer, Waltham, MA). CXCL10 at 3 nM was used to stimulate migration of cells through a filter containing 5 µm pores on Migration Plates from Neuro Probe (Gaithersburg, MD; Cat. ChemoTx 106-5). A titration of BMS-936557 from 13 pM to 600 nM was added to the cells. CXCL10 without antibody was used to establish maximal migration. Migration toward media alone without CXCL10 was used to measure background migration.

Following 2 hour incubation at 37 °C, migrated cells were detected by addition of DELFIA Europium solution to the lysed cells and detected by time resolved fluorescence on the Fusion (Perkin Elmer). Data was graphed and analyzed with GraphPad Prism software, using nonlinear regression and sigmoidal dose-response curves.

Statistics. Samples were evaluated in a randomized manner by three independent investigators (PJ, NS and KM) who were blinded to the treatment conditions. Data are presented as means +/- standard error (SE) and were analyzed by paired, Student's t test or by analysis of variance with a Bonferroni correction for multiple group comparisons.

RESULTS

Anti-CXCL10 antibodies block migration and calcium flux and have no effect on weight gain or food intake in mice.

Generated antibodies used in this study recognize human (BMS-936557) and mouse CXCL10. To confirm the effect of the human and mouse antibodies, migration and calcium flux assays were performed. Anti-human CXCL10 antibody blocked migration (Suppl.Fig.1A) and calcium flux (Suppl.Fig.1B) of 300.19 cells transfected with human CXCR3 with an IC₅₀ of 0.49 and 0.22 nM, respectively. When migration and calcium flux were measured using mouse CXCL10, the anti-mouse CXCL10 antibody had an IC₅₀ of 32 (Suppl.Fig.1C) and 36 nM (Suppl.Fig.1D), respectively. In contrast, human and mouse IgG1 isotype controls had no effect.

To assess the effect of specifically inhibiting CXCL10 during diabetes progression, we fed 6-week old C57BL/6J mice a normal (ND) or high fat/high sucrose diet ("Surwit", HFD [37, 41]) and either injected peritoneally twice weekly an anti-CXCL10 antibody or

IgG isotype control for 16 weeks. Mice fed HFD gained more weight than the ND group, which reached significance already at 2 weeks (Fig.1A). The anti-CXCL10 antibody had neither an effect on food intake (Fig.1B) nor on weight gain (Fig.1A).

Anti-CXCL10 improves glucose tolerance, insulin secretion and insulin sensitivity in mice.

Insulin and glucose tolerance together with insulin secretion was measured after 4, 8, 12 and 16 weeks of diet. Starting at 4 weeks on diet, untreated HFD mice developed glucose intolerance as measured by glucose tolerance test (ipGTT), while anti-CXCL10 treated mice had significantly lower glucose levels (not shown). After 12 weeks of diet, HFD mice developed elevated fasting glucose levels relative to ND mice (132 ± 3 mg/dl for untreated HFD control mice vs. 66 ± 3 mg/dl for ND control mice, $p<0.05$). This was prevented by anti-CXCL10 treatment in the HFD group (102 ± 4 mg/dL, $p<0.05$). Anti-CXCL10 improved glucose tolerance after 4, 8, 12 and 16 weeks of treatments and feeding. Fig.2A shows the results of the glucose tolerance tested after 16 weeks, HFD feeding increased glucose levels significantly at all time points, while anti-CXCL10 treated mice show significantly improved glycemia. Area under the curve analysis revealed significantly lower glucose levels in the anti-CXCL10 treated HFD mice (Fig.2B, $p<0.05$).

Since inhibition of CXCL10 led to an improvement in glycemic control, we tested the effect of anti-CXCL10 on insulin secretion. Fasted mice were injected with 2 g/kg glucose, and insulin secretion was measured before (0 min) and 30 min after glucose injection. In parallel to the HFD induced hyperglycemia (Fig.2A), mice were also hyperinsulinemic at the basal state (Fig.2C), compared to ND mice. After 16 weeks of

HFD, fasting insulin levels were 2.4-fold higher in untreated HFD mice, compared to ND (Fig.2C, $p<0.05$), glucose stimulated insulin secretion was almost fully abolished in this group, while anti-CXCL10 treatment in HFD mice restored GSIS (3.9-fold increased stimulatory index compared to untreated HFD control, $p<0.001$, Fig.2D), and also basal insulin levels were normalized (Fig.2D, 4.3-fold difference, $p<0.001$).

Since the normalization of fasting insulin levels by anti-CXCL10 treatment in HFD mice could be a result of improved insulin sensitivity, we measured insulin tolerance in an ipITT (Fig.2E) and applied the analysis of homeostasis model assessment measure of insulin resistance HOMA-IR (Fig.2F).

No significant differences in glucose levels were observed between treated and untreated animals of the ND group before and after the ipITT (Fig.2E). Untreated HFD-mice showed elevated glucose levels throughout the test compared to ND mice ($p<0.05$, Fig.2E at 0,15 and 30 min). In contrast, in the anti-CXCL10 treated HFD mice, glucose levels were unchanged compared to ND mice before and after the insulin injection

HOMA-IR shown as the product of fasting insulin and fasting glucose [36] was elevated by the HFD from 4 weeks on, which gradually increased with length of diet (1.7-fold after 4 weeks and 4.3-fold increase in HFD compared to ND control, Fig.2F). Anti-CXCL10 treatment significantly reduced HOMA-IR at all time points (Fig.2F, 3.7-fold decrease after 16 weeks).

Anti-CXCL10 improves β -cell survival, restores β -cell mass and reduces intra-islet cytokine expression.

We hypothesized that CXCL10 neutralization protects pancreatic β -cells and analyzed β -cell apoptosis, β -cell mass and expression of pro-inflammatory cytokines in islets.

Previously, we reported increased β -cell mass in mice after 12 weeks of HFD despite the tendency for increased β -cell apoptosis at that time-point [37]. This was again confirmed in this study; HFD feeding induced a compensatory increase in β -cell mass in both control and anti-CXCL10 treated HFD groups (1.5- and 1.8-fold increase in both HFD control and HFD anti-CXCL10 groups vs. the respective ND groups, $p < 0.05$, Fig.3A). In contrast to the findings after 12 weeks on diet, no increase in β -cell mass was observed in control mice after 16 weeks on HFD compared to those on ND (Fig.3B), but anti-CXCL10 still restored β -cell mass compensation in HFD mice (2.3-fold increase vs. HFD non-treated, 1.7-fold increased vs. ND anti-CXCL10 treated). Such β -cell protection was also mirrored by TUNEL staining of pancreatic sections. HFD feeding for 16 weeks induced a 4.1-fold induction of TUNEL-positive β -cells, compared to ND ($p < 0.01$), which was prevented by anti-CXCL10 treatment (2.7-fold reduction compared to HFD alone, $p < 0.01$, Fig.3C,D).

Anti-CXCL10 decreases pro-inflammatory cytokine expression in pancreatic islets, liver and fat

Obesity and diabetes are highly correlated with increased inflammation; cytokines induce their own expression through an intracellular signaling cascade. Therefore, we tested whether inhibition of the chemokine CXCL10 by anti-CXCL10 treatment may inhibit expression of the major cytokine produced during obesity, IL-1 β , IL-6 and TNF α as well as CXCL10 itself in islets, liver, fat and hypothalamus.

CXCL10 expression was highly increased by HFD feeding over 16 weeks in islets, liver and adipose tissue, and anti-CXCL10 treatment normalized CXCL10 mRNA expression in all 3 tissues (Fig.4A,C,D). In islets, CXCL10 expression in the β -cell was confirmed

by immunocytochemistry, but we only detected few CXCL10-positive cells in each pancreas from the HFD group, and such CXCL10-positive cells were never observed in ND or anti-CXCL10 treated ND and HFD groups (Fig.4B). The cytokines IL-1 β and IL-6 were also upregulated by the high fat diet in islets, and also tend to increase in fat and liver and were normalized by anti-CXCL10, with the exception of IL-1 β , which was not reduced by anti-CXCL10 in the liver (Fig.4D). TNF α was not increased by the HFD in islets, but was upregulated in liver and fat, where it was also normalized by anti-CXCL10. Unexpectedly, neither HFD feeding nor anti-CXCL10 treatment induced any cytokine expression in the hypothalamus (Fig.4E). Such negative results were confirmed in additional sets of experiments in mice fed a HFD, and we again failed to measure any changes in cytokine mRNA expression in the hypothalamus (data not shown).

Eventually, we tested the anti-CXCL10 antibody in human islets for its ability to reduce CXCL10 expression (Fig.4F). CXCL10 mRNA was upregulated by the diabetogenic milieu of glucose and palmitate, this was prevented by pre-treatment of human islets with anti-CXCL10 (Fig.4F).

DISCUSSION

MDX-1100, a highly specific CXCL10 neutralizing antibody blocks CXCL10 induced calcium flux and cell migration with an estimated IC₅₀ in the low nM range [42] has entered clinical trials for the treatment of two classical inflammatory diseases, ulcerative colitis (UC) [42] and rheumatoid arthritis, where MDX-1100 was well-tolerated, demonstrated clinical activity and no drug related serious adverse events were reported. It specifically inhibits CXCL10 and not CXCL9 or CXCL11, the other 2 ligands of the

classical receptor for CXCR3 [42].

Based on our previous data showing CXCL10 as a major cytokine produced by pancreatic islets in response to a diabetic milieu in T1D as well as in T2D, we tested the mouse specific anti-CXCL10 antibody for its efficacy to protect from diabetes progression in the high fat/ high sucrose fed mouse model. Anti-CXCL10-antibody improved glycemia, insulin secretion, insulin sensitivity and β -cell survival and function. Also, CXCL10 mRNA levels as well as inflammatory genes produced downstream the TLR4-NF κ B pathway, which were increased in response to the HFD in pancreatic islets, in fat and liver, were significantly decreased by the anti-CXCL10 treatment.

Many studies show TLR4 activation in response to free fatty acids [12, 13, 43] in fat, brain as well as in pancreatic islets, which supports the link of obesity and metabolic diseases, where inflammation occurs in insulin sensitive tissue and promotes insulin resistance as well as in islets, where β -cell failure occurs. Thus TLR4 antagonism has been identified as a potent target to improve glucose metabolism [12], although TLR4 was also shown to contribute to the proinflammatory state in models of T1D [44]. Direct binding of FFA to TLR4 has not been identified, but a recent study suggests fetuin as a binding partner of FFA to TLR4 [45]. In line with this observation, fetuin-A levels are associated with a higher risk of developing T2D [46]. The present study clearly shows HFD induced cytokine and chemokine production in insulin sensitive tissue and in pancreatic islets. The fact that neutralization of the chemokine CXCL10 normalizes glycemia suggests that the activation of the inflammatory response is causative for the development of insulin resistance and impaired β -cell function. Cytokines and chemokines are produced downstream of TLR4. This opens a vicious cycle when chronically produced in obesity

[23]. CXCL10 was thereby most prominently induced in islets in HFD treated mice and its production totally reduced by CXCL10 antagonism, supporting the effect of its own production-stimulation. Although we have observed a >10-fold induction of CXCL10 mRNA in isolated islets, immunostaining of the pancreas only revealed in few β -cells positively stained for CXCL10. Such CXCL10 positive β -cells were only detected in islets from the HFD fed mice, and expression pattern is very similar to CXCL10 expressing β -cells in patients at recent onset of T1D [30]. One explanation could be that CXCL10 is secreted and thus could not always be detected by immunohistochemistry.

IL-1 β and IL-6 were also highly induced in islets by HFD feeding, both shown to be implicated in the regulation of glycemia [47, 48], while TNF α levels were not affected, which is in line with previous observations in human islets in vitro [6]. CXCL10 neutralization led to reduced production of IL-1 β , IL-6, most likely through a feed-back mechanisms, which reduces CXCL10 induced signalling.

Analysis of insulin sensitive tissue revealed HFD-induced production of CXCL10, IL-1 β , IL-6 and TNF α in fat and liver, which was significantly reduced by CXCL10 antagonism. Only in the liver, IL-1 β production failed to decrease, pointing to a CXCL10 independent effect.

In contrast, we did not detect changes in inflammatory cytokine profile in response to HFD feeding in the hypothalamus and thus, also no effect was seen by CXCL10 antagonism. In contrast, previous studies show upregulation of inflammatory gene expression including CXCL10 in the hypothalamus after 20 weeks HFD feeding. Here, a different diet, rich in fat but not in glucose was used. Also, hypothalamic NF κ B activation was shown in numerous elegant studies in response to obesity [49]. Inhibition of NF κ B signalling

improved glycemia and insulin sensitivity, but downstream NF κ B targets were not investigated in these studies [49]. These previous studies support an involvement of the hypothalamus in the regulation of glycemia, but cytokine expression occurs in waves in response to the HFD [50]. Although inflammatory cytokines are increased in obesity and were measured in numerous studies in serum of overweight and obese patients as a predictive marker for the development of T2D, it remains still largely unknown if cytokines, especially IL-1 β , which mainly functions locally in the inflamed tissue, contribute to the inter-organ crosstalk in obesity. Despite high levels of IL-1 β and CXCL10 mRNA in the insulin sensitive tissue, we could only measure marginal levels in the serum, which were mostly under the detection limit of the assays and thus, no significant differences were obtained. Such measures would support a local and no systemic role of cytokines/ chemokines in sensitive tissue. Nevertheless, because of the presence of soluble IL-1 β binding proteins and its rapid degradation despite immediate freezing of the serum [51], we cannot exclude false-negative measures in the mouse serum.

In summary, with the robust effect of the anti-CXCL10 antibody on glycemia, insulin secretion and β -cell survival, we provide here a novel target for the treatment of T2D. The effect of CXCL10 neutralization *in vitro* in human islets and *in vivo* in experimental models of T1D supports the protection on the level of the β -cell [52, 53].

Acknowledgements

Funding. This work was supported by the German Research Foundation (DFG, MA4172/1-1), the JDRF, BMBF and the ERC. Human islets were provided through the

JDRF award 31-2008-413 (ECIT Islet for Basic Research program). Pina Cardarelli is employee of Bristol-Myers Squibb.

Authors' contribution: Designed, performed and analysed research: PS, NS, PC, KM.

Performed experiments: JB, PJ. Contributed new reagents or analytic tools: LG, TB.

Wrote paper: PS, KM.

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FIGURE LEGENDS

Figure 1. CXCL10 neutralization does not influence weight gain

Mice were fed a normal (ND) or high fat diet (HFD) and injected twice weekly with either IgG (control) or anti-CXCL10 at 10 mg/kg for 16 weeks. Body weight/mouse (A) and average weekly food intake/5 mice in a cage (B) are from 3 independent experiments from 13 mice/group. Data show mean \pm SE. * $p < 0.05$ HFD vs. ND, same treatment

Figure 2. CXCL10 neutralization improves insulin secretion and sensitivity in mice.

Mice were fed a normal (ND) or high fat diet (HFD) and injected twice weekly with either IgG (control) or 10 mg/kg anti-CXCL10 for 16 weeks. **(A)** Glucose was measured during a glucose tolerance test (ipGTT) after 16 weeks before (0 min) and after glucose injection (1g/kg BW) and area under the curve **(B, AUC)** of the glucose tolerance tests calculated for each treatment group during the 16 weeks of diet and treatment. **(C)** Insulin secretion was measured during the ipGTT before (0 min) and after glucose injection (30 min, 2g/kg BW) and **(D)** the insulin stimulatory index calculated as a ratio of stimulated (30 min) over basal (0 min) insulin. **(E)** Insulin tolerance test (ipITT) with an intraperitoneal injection of 0.75 U/kg insulin after 5 h fast performed after 16 weeks and **(F)** Homeostatic Model Assessment–Insulin Resistance (HOMA-IR) of mice as a product of fasting insulin and glucose levels during 16 weeks of diet and treatment. Data are means \pm SE from 3 independent experiments from 13 mice/group. * $p < 0.05$ HFD vs. ND, same treatment, ** $p < 0.05$ anti-CXCL10 vs. IgG, same diet

Figure 3. CXCL10 neutralization restores β -cell mass and improves β -cell survival.

(A,B) β -cell mass per pancreas was calculated as the product of the relative cross-sectional area of β -cells (determined by quantification of the cross-sectional area occupied by β -cells divided by the cross-sectional area of total tissue) and the weight of the pancreas from the mice after 12 **(A)** and 16 **(B)** weeks of diet. 10 sections per mouse spanning the width of the pancreas were included in the analysis. **(C,D)** Triple staining for TUNEL in red, insulin in green and DAPI in blue was performed on fixed, paraffin embedded islet sections (Magnification x250). White arrows indicate TUNEL positive β -

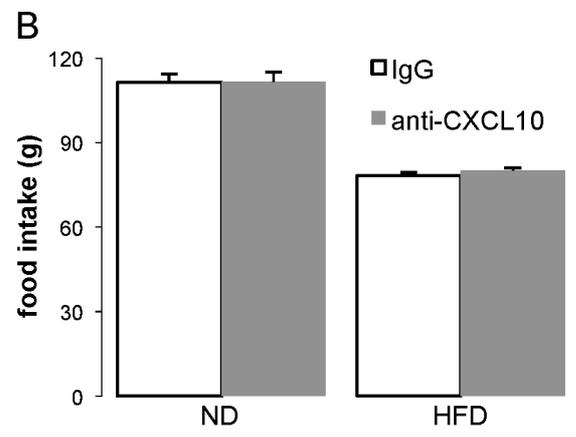
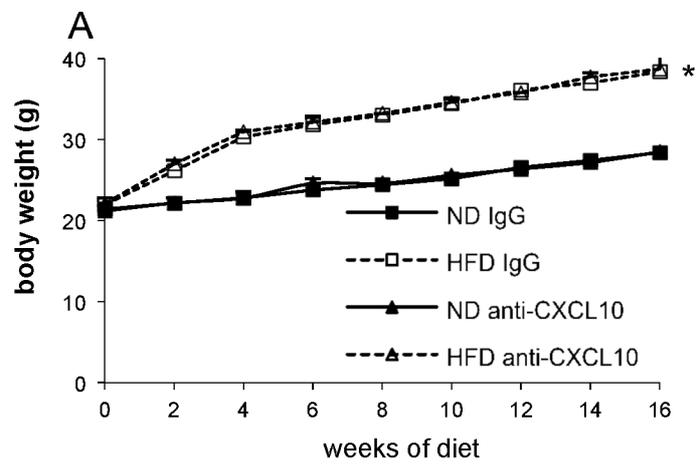
cells (triple-stained, **D**). Results are expressed as percentage of TUNEL-positive β -cells \pm SE. The mean number of β -cells scored was 10,790 for each treatment condition (**C**).

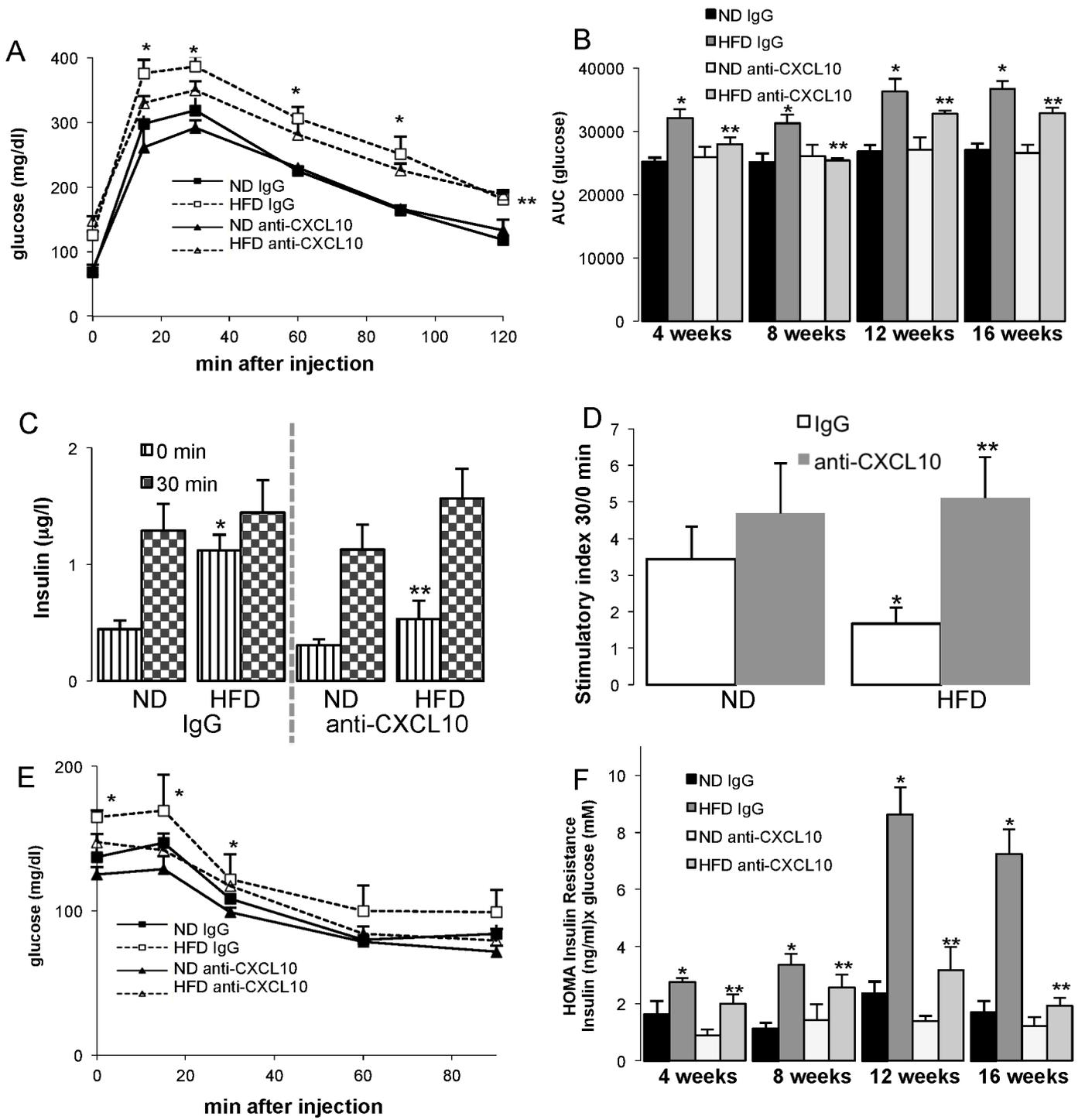
* $p < 0.05$ HFD vs. ND, same treatment, ** $p < 0.05$ anti-CXCL10 HFD vs. IgG HFD.

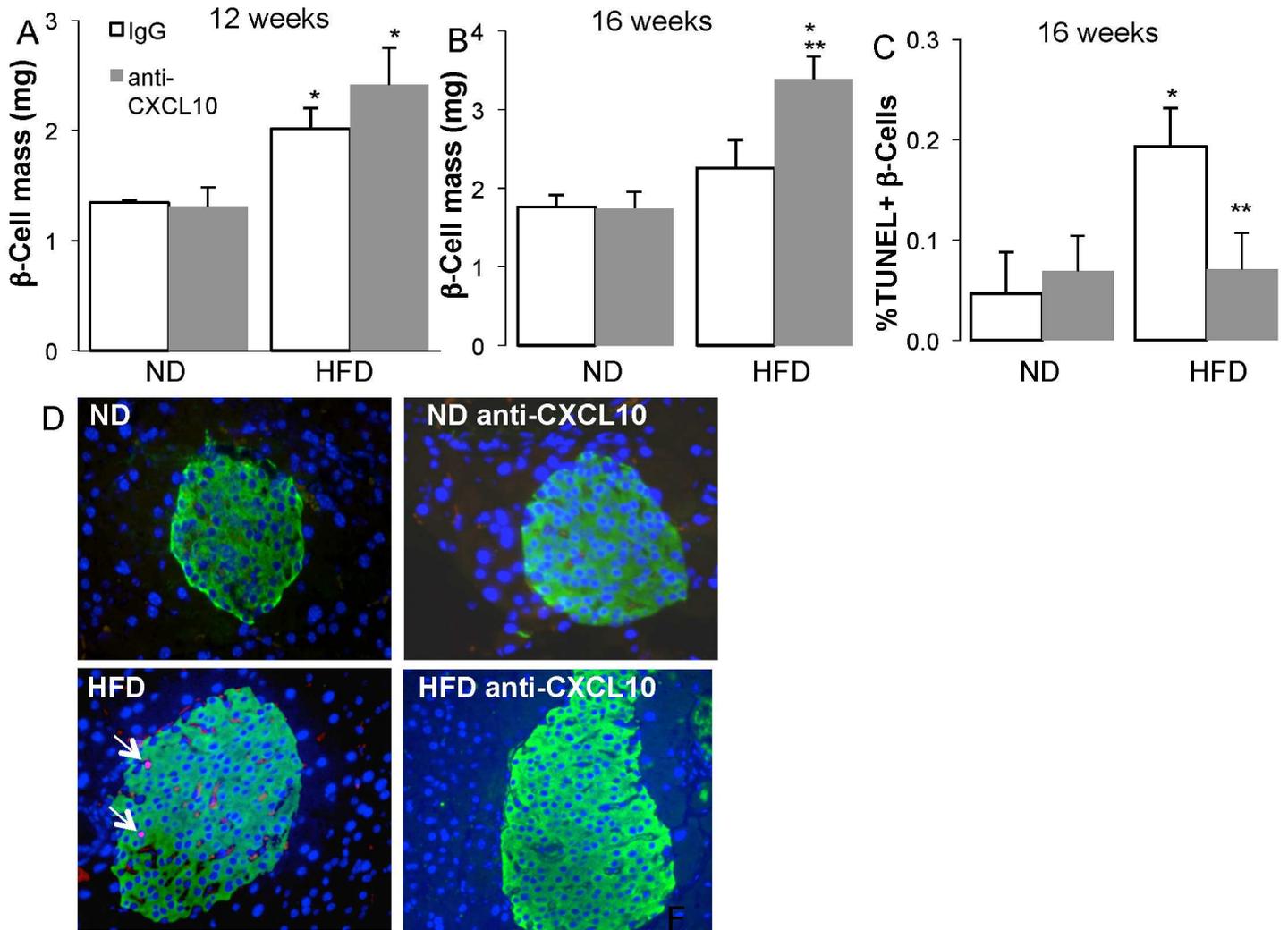
Figure 4. Anti-CXCL10 decreases pro-inflammatory cytokine expression in pancreatic islets, liver and fat

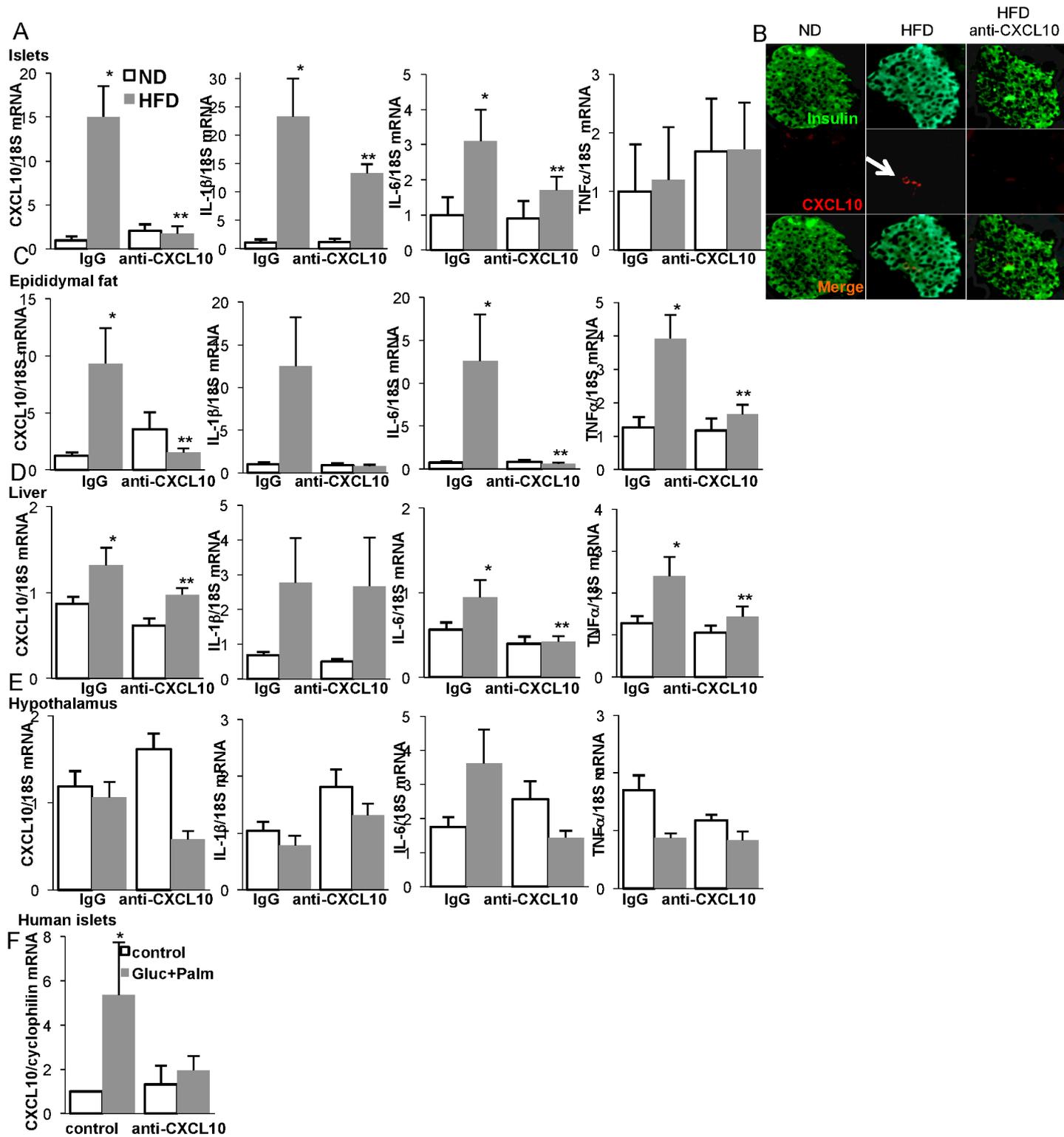
(**A,C,D,E,F**) mRNA expression of CXCL10, IL-1 β , IL-6 and TNF α in isolated islets (**A**), epididymal fat (**C**), liver (**D**) and hypothalamus (**E**) after 16 weeks of diet and treatment with IgG or anti-CXCL10 analyzed by RT-PCR. Data are means of the relative quantification normalized to one control ND mouse, respectively \pm SE from 9 (**C-F**) or 3 (**A**) mice/group. * $p < 0.05$ HFD vs. ND, same treatment, ** $p < 0.05$ anti-CXCL10 vs. IgG, same diet. (**B**) CXCL10 expression in islets was confirmed by immunocytochemistry of mouse pancreases from the 4 treatment groups stained for insulin in green and CXCL10 in red. The white arrow points to CXCL10 positive β -cells.

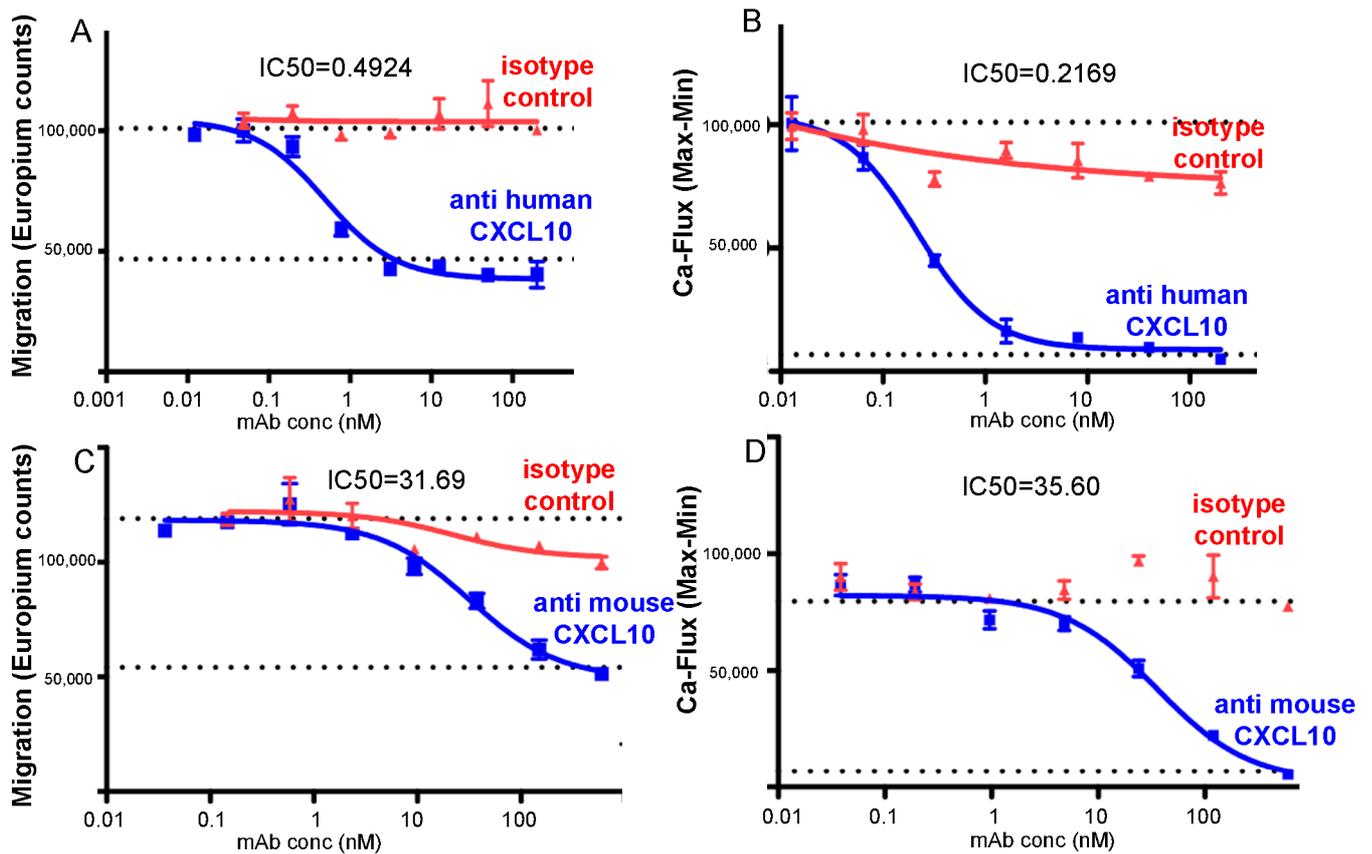
(**F**) Human islets were exposed to the mixture of elevated glucose (22.2mM) and palmitate (0.5mM) for 3 days and treated with anti-CXCL10-Ab (10 μ g/ml). mRNA expression of CXCL10 was analyzed by RT-PCR. Data are means of the relative quantification normalized to untreated control islets from 4 independent experiments from 4 different human islet donors \pm SE.











Suppl.Fig.1. Anti-CXCL10 antibodies block migration and calcium flux.

(A,C) Cells were loaded with BATDA. CXCL10 at 3 nM was used to stimulate migration of cells through a filter containing 5 μ m pores on Migration Plates from Neuro Probe (Gaithersburg, MD; Cat. ChemoTx 106-5). A titration of BMS-936557 from 13 pM to 600 nM was added to the cells. CXCL10 without antibody was used to establish maximal migration. Migration toward media alone without CXCL10 was used to measure background migration. Following 2 hour incubation at 37 $^{\circ}$ C, migrated cells were detected by addition of Europium solution to the lysed cells and detected by time resolved fluorescence on the Fusion (Perkin Elmer). Data was graphed and analyzed with GraphPad Prism software, using nonlinear regression and sigmoidal dose-response curves.

(B,D) Cells were loaded with FLIPR Calcium 4 dye (Molecular Devices, Sunnyvale CA). CXCL10 at 3 nM was used to stimulate calcium flux. A titration of BMS-936557 from 13 pM to 600 nM was used to inhibit the response. A maximal calcium response was set with CXCL10 minus antibodies. A baseline response was established with buffer stimulation of cells without CXCL10. Calcium fluxes were read on the Flexstation (Molecular Devices, Sunnyvale, CA). Data was graphed and analyzed with GraphPad Prism software, using nonlinear regression and sigmoidal dose-response curves.

Supplemental data:

Mouse specific primers of the following sequences were designed to perform RT-PCRs:

Tubulin - product length: 250 bp
Forward: GTTGGCCAGGCTGGTGTCCAG
Reverse: CTGTGATGAGCTGCTCAGGGTGG

Cyclophilin - product length: 210 bp
Forward: GTGGTCTTTGGGAAGGTGAA
Reverse: TTACAGGACATTGCGAGCAG

CXCL10 - product length: 158 bp
Forward: GTGAGAATGAGGGCCATAGG
Reverse: TTTTGGCTAAACGCTTTCATT

IL6 - product length: 88 bp
Forward: CCAGAGATACAAAGAAATGATGG
Reverse: ACTCCAGAAGACCAGAGGAAAT

IL1 β - product length: 249 bp
Forward: CAGGCAGGCAGTATCACTCA
Reverse: AGCTCATATGGGTCCGACAG

TNF α - product length: 138 bp
Forward: ACGCCATGGATCTCAAAGAC
Reverse: AGATAGCAAATCGGCTGACG

Human specific TaqMan (Applied Biosystems) assays were used to perform RT-PCRs:

CXCL10: Hs00171042_m1
cyclophilin: Hs99999904_m1

The DPP-4 inhibitor Linagliptin restores beta-cell function and survival in human isolated islets through GLP-1 stabilization

My contribution: Performed islet treatment and GSIS. RT-PCR and microscopic analysis

The DPP-4 Inhibitor Linagliptin Restores β -Cell Function and Survival in Human Isolated Islets Through GLP-1 Stabilization

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Context: Inhibition of dipeptidyl peptidase-4 (DPP-4) is a potent strategy to increase glucose-dependent insulinotropic polypeptide and glucagon like peptide 1 (GLP-1) induced insulin secretion in diabetes. It is important to know whether new drugs approved for the treatment of type 2 diabetes have direct effects on the β -cell.

Objective: Herein we investigated the effect of linagliptin, a novel DPP-4 inhibitor, on β -cell function and survival.

Design: Human islets were exposed to a diabetic milieu (11.1–33.3 mM glucose, 0.5 mM palmitate, the mixture of 2 ng/mL IL-1 β +1000 U/mL interferon- γ , or 50 μ M H₂O₂) with or without 500 ng/mL IL-1 receptor antagonist (IL-1Ra) or 30–50 nM linagliptin.

Results: Linagliptin restored β -cell function and turnover, which was impaired when islets were exposed to elevated glucose, palmitate, cytokines, or H₂O₂. Pretreatment with IL-1Ra was similarly effective, except against H₂O₂ treatment. Nitrotyrosine concentrations in islet lysates, an indicator of oxidative stress, were highly elevated under diabetic conditions but not in islets treated with linagliptin or IL-1Ra. Linagliptin also reduced cytokine secretion and stabilized GLP-1 in islet supernatants.

Conclusions: We show that the novel DPP-4 inhibitor linagliptin protected from gluco-, lipo-, and cytokine-toxicity and stabilized active GLP-1 secreted from human islets. This provides a direct GLP-1 mediated protective effect of linagliptin on β -cell function and survival. (*J Clin Endocrinol Metab* 98: E1163–E1172, 2013)

Dipeptidyl peptidase 4 (DPP-4) is a ubiquitous cell-membrane protein enzyme responsible for cleaving and inactivating both incretins, glucagon like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (1), which are released by cells in the small intestine after the ingestion of food and which potentiate glucose-stimulated insulin secretion (2). Patients with type 2 diabetes (T2D) have

impaired insulin secretion and chronic hyperglycemia, and the effect of the incretins is significantly reduced (3).

Incretin-based therapies such as injectable GLP-1 receptor agonists or DPP-4 inhibitors are established treatments for T2D because of their glucose-dependent stimulation of insulin secretion, their inhibition of glucagon secretion, and their intrinsic lack of risk for hypoglycemia

ISSN Print 0021-972X ISSN Online 1945-7197

Printed in U.S.A.

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Received January 1, 2013. Accepted April 22, 2013.

First Published Online April 30, 2013

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Abbreviations: DPP-4, dipeptidyl peptidase 4; ECM, extracellular matrix; GLP-1, glucagon like peptide 1; GSIS, glucose-stimulated insulin secretion; IFN, interferon; IL-1Ra, IL-1 receptor antagonist; KRB, Krebs-Ringer bicarbonate buffer; SDF1 α , stromal cell-derived factor-1 α ; T2D, type 2 diabetes; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick-end labeling;

(4), although an increased risk for pancreatitis has been discussed recently (5).

Long-term clinical effects of incretin-based drugs on the β -cell are still speculative (6). However, numerous *in vivo* and *in vitro* studies in rodents show that GLP-1 increases β -cell mass, proliferation (7–9), and β -cell neogenesis (10). GLP-1 inhibits β -cell apoptosis in human islets (11–13), and a protective effect was also observed in islets isolated from patients with T2D (14).

Accordingly, stabilizing GLP-1 by DPP-4 inhibition restores glycemic control in diabetic animal models (2) and improves β -cell survival, replication, and neogenesis in the diabetic mouse and rat streptozotocin model (15, 16) and in the VDF diabetic rat (17).

The direct effect of DPP-4 inhibitors on human β -cells has not been investigated yet. Thus, the aim of this study was to test whether a DPP-4 inhibitor would restore β -cell function and survival under diabetogenic conditions in human islets and whether cytokine production from islets under such conditions is affected. For our studies, linagliptin, a xanthine-based, highly potent, and long-acting DPP-4 inhibitor was used, which has recently been approved for the treatment of T2D (18, 19). IL-1 receptor antagonist (IL-1Ra), which improves β -cell survival and function by neutralizing IL-1 β (20, 21), was used as a positive control for a β -cell protective effect. In the present study, we provide evidence that linagliptin improves β -cell survival and function in human islets through stabilization of GLP-1, which is secreted during islet culture.

Materials and Methods

Cell culture

Human islets were isolated from 8 pancreata of healthy organ donors at Lille University and cultured in CMRL-1066 medium as described previously (22, 23). Islet purity was greater than 95% as judged by dithizone staining (if this degree of purity was not achieved by routine isolation, islets were handpicked). For long-term *in vitro* studies (96 hours), islets were cultured on extracellular matrix-coated plates derived from bovine corneal endothelial cells (Novamed Ltd, Jerusalem, Israel), allowing the cells to attach to the dishes and spread, preserving their functional integrity (24). In parallel, islets were cultured in suspension dishes, treated for 72 hours, and fixed with Bouin's solution. Islet sections were prepared as described previously (21).

Human islets were exposed to a diabetic milieu [11.1–33.3 mM glucose or 5.5 mM glucose+0.5 mM palmitic acid, the mixture of 2 ng/mL recombinant human IL-1 β (R&D Systems, Minneapolis, Minnesota)] +1000 U/mL recombinant human interferon (IFN)- γ (Pepro Tech, Rocky Hill, New Jersey) for 72 hours or 50 μ M H₂O₂ for 8 hours with or without 500 ng/mL IL-1Ra, 10 nM-1 μ M linagliptin, 200 nM sitagliptin, 100 nM exendin-(9–39) (Bachem, Bubendorf, Switzerland) or 0.5 nM GLP-1 (Sigma, St Louis, Missouri). Compounds were added 1 hour before the exposure to the diabetogenic conditions and

throughout the culture. Palmitic acid was dissolved as described previously (25).

Ethical approval for the use of islets had been granted by the Ethical Committee of University of Bremen. We received the islets from the European Consortium For Islet Transplantation. Whenever an islet isolation fails to be suitable for transplantation, centers provide them for islet research. Thus, these research projects comply with National Institutes of Health regulation Public Health Service 398, exemption 4. Human pancreata were harvested from brain dead donors, according to the European and national regulations for organ procurement. Donors or their family members gave written consent to donate organs for transplantation and research, all documented by the transplantation centers. Human islet isolations were performed with the approved protocols of the centers.

Cell turnover

For detection of β -cell apoptosis, 100 human islets were cultured in suspension dishes, treated for 72 hours, and fixed with Bouin's solution. Islet sections were prepared as described previously, deparaffinized, rehydrated, and incubated with 20 mg/mL proteinase K (Roche Applied Science, Mannheim, Germany) for 15 minutes at 37°C before staining. In parallel, islets on extracellular matrix (ECM)-coated dishes were fixed using 4% paraformaldehyde and insulin costained with apoptosis analyzed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick-end labeling (TUNEL) technique according to the manufacturer's instructions (in situ cell death detection kit, TMR red; Roche Applied Science) (26).

For the analysis of β -cell proliferation, fixed islets on ECM-coated dishes were incubated overnight at 4°C with mouse anti-human Ki67 (Invitrogen, Carlsbad, California), followed by detection with donkey-rat Cy3-conjugated antibody (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania). Insulin was analyzed by anti-guinea pig insulin antibody (Dako, Glostrup, Denmark) followed by detection with donkey anti-guinea pig fluorescein isothiocyanate-conjugated antibody (Jackson ImmunoResearch Laboratories). Islets and islet sections were embedded in Vectashield mounting medium (Vector laboratories, Burlingame, California). Fluorescence was analyzed using a Nikon Eclipse Ti-U inverted research microscope (Nikon GmbH, Dusseldorf, Germany), and images were acquired using NIS-Elements software (Nikon).

Glucose-stimulated insulin secretion (GSIS)

Islets used to perform glucose-stimulated insulin secretion experiments were kept in culture medium on matrix-coated dishes. For each independent experiment, 20 islets were plated and exposed to the treatment conditions as indicated above. For acute insulin release, islets were washed and preincubated (30 minutes) in Krebs-Ringer bicarbonate buffer (KRB) containing 2.8 mM glucose. The KRB was then replaced by KRB containing 2.8 mM glucose for 1 hour (basal), followed by an additional 1 hour incubation in KRB containing 16.7 mM glucose (stimulated). Islets were lysed in lysis buffer and whole islet protein measured by bicinchoninic acid protein assay (Pierce, Rockford, Illinois/Thermo Scientific, Rockford, Illinois). Insulin was determined using a human insulin ELISA kit (ALPCO, Windham, New Hampshire) and normalized to whole-islet cell lysate protein content.

Determination of nitrotyrosine

Nitrotyrosine concentration in human islets after the 4-day culture period was determined in islet cell lysates by an adapted ELISA method as previously described (27, 28). At the end of the incubation, islets (a total of 30 islets/condition) were washed in PBS, suspended, and lysed for 40 minutes on ice in lysis buffer [20 mM Tris acetate (pH 7.0), 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1% Triton X-100, 5 mM sodium pyrophosphate, 10 mM β -glycerophosphate (all Sigma Aldrich)] containing $1\times$ protease and a phosphate inhibitor cocktail (Thermo Scientific). Protein concentrations of islet cell lysates were determined by bicinchoninic acid protein assay, and whole-cell lysates were stored at -80°C until assayed. The standard curve samples were prepared by dissolving 1 mg/mL BSA (Sigma) in 50 mmol/L KH_2PO_4 . After the addition of 1 mmol/L tetranitromethane, the dilution was incubated for 30 minutes at 37°C and adjusted to pH 10, and the amount of nitrotyrosine present was measured at 430 nm, and moles nitrotyrosine for each mole protein were calculated directly using the Beer-Lambert law ($\epsilon = 4300 \text{ M}^{-1}\text{cm}^{-1}$) (29). Standards of 0.05–0.6 pmol/ μL were prepared. Maxisorp ELISA plate (number 353279; BD, Franklin Lakes, New Jersey) was coated with standards and samples in coating buffer (50 mmol/L Na_2CO_3 - NaHCO_3 buffer at pH 9.6) overnight at 4°C , washed 6 times with 0.05% Tween 20 in PBS, and incubated with IgG monoclonal antinitrotyrosin (Upstate Biotechnology, Lake Placid, New York/Millipore, Bedford, Massachusetts) for 2 hours at room temperature, washed, and incubated for 45 minutes at 37°C with horseradish peroxidase-conjugated goat antimouse (Zymed, San Francisco, California) and washed again. For the peroxidase reaction, 100 μL tetramethyl-benzidine microwell peroxidase substrate (Sigma) was added for 10 minutes at room temperature, and the reaction was stopped by adding 50 μL stop solution (BD Opty Kit B/0.5 M H_2SO_4 , BD) and read at 492 nm on a microplate reader. Concentration of nitrotyrosine was normalized to whole-protein content of islet lysates.

Determination of cytokines, GLP-1, and DPP-4

After the 4-day culture period, human islet supernatants were measured for cytokine release using the human proinflammatory tissue culture kit (Meso Scale Discovery; Rockville, Maryland). Total and active human GLP-1 was detected by the same assay platform (Meso Scale Discovery).

DPP-4 activity was detected using H-Ala-Pro-7-amido-4-trifluoromethylcoumarin (purchased from Bachem) in a 200-mM stock solution in dimethylformamide, which was diluted 1000-fold with water before the assay. The assay itself was performed in black flat-bottom, 96-well plates by mixing 50 μL of the diluted substrate (final concentration in the assay 100 μM), 25 μL of supernatant, and 25 μL of assay buffer (100 mM Tris-HCl; 100 mM NaCl, pH 7.8). The plate was then incubated at room temperature for 1 hour and fluorescence was measured at excitation/emission wavelengths of 405/535 nm. For standards, 20 μL of appropriate compound dilutions was prepared in assay buffer (compound stock solutions in dimethylsulfoxide final concentration in the assay 1%).

RNA extraction and RT-PCR analysis

Total RNA was isolated from cultured human islets by the Trizol method (peqGOLD TriFast; PeQLab, Erlangen, Germany). For quantitative analysis, we used the Applied Biosys-

tems StepOne real-time PCR system (Applied Biosystems, Foster City, California) with a commercial kit [Power SYBR Green PCR master mix or TaqMan(R) Fast Universal PCR master mix for TaqMan assays; Applied Biosystems]. Primers used were 5'-ggctgcatatggaggta-3'/5'-cagggtcttgagatctgag-3' (DPP-4), 5'-agagccaactgcaagcatct-3'/5'-ctttagctcgggtcaatgc-3' [stromal cell-derived factor-1a (SDF1 α)], 5'-tccccactctgagttcac-3'/5'-acatctcgacgcatctcag-3' (IL-6), 5'-tcctctcgcttcagacac-3'/5'-gtaattggccgccacacagt-3' (GLP-1 receptor) 5'-gttggccaggctggtgccag-3'/5'-ctgtgatgagctgctcagggtgg-3' (tubulin), 5'-ccaaccgagaagatga-3'/5'-ccagaggctacaggatag-3' (actin), 5'-tacgggtctggcatctgtt-3'/5'-ccattgtgtgggtccagc-3' (cyclophilin), and 5'-aaacggctaccatccaag-3'/5'-cctccaatggatcctgta-3' (18S). The TaqMan(R) gene expression assays (Applied Biosystems) were used for IL-1 β , IL-8, cyclophilin, and tubulin.

Statistical analysis

Immunostainings were evaluated in a randomized manner by 2 investigators (P.S., S.L.), who were blinded to the treatment conditions. Data are presented as means \pm SEM and were analyzed by a paired Student's *t* test or by ANOVA with a Bonferroni correction for multiple group comparisons.

Results

Linagliptin, sitagliptin, and IL-1Ra improve β -cell survival

Loss of β -cells by apoptosis and decreased β -cell mass have been recognized as major pathological factors for the progression of diabetes. We tested the hypothesis whether linagliptin can protect β -cells from damage induced by diabetogenic conditions. Isolated human pancreatic islets were exposed for 4 days to increasing glucose concentrations (5.5, 11.1, 33.3 mM), 0.5 mM palmitate, the mixture of cytokines (2 ng/mL IL-1 β +1000 U/mL IFN- γ), or 50 μM H_2O_2 , for 8 hours, conditions that caused β -cell apoptosis and almost complete loss of proliferation. Islets were pretreated with 500 ng/mL IL-1Ra, which has been shown to improve β -cell survival (21), or with 50 nM linagliptin, which was added 1 hour before the diabetogenic conditions and maintained throughout the 4-day culture. At basal culture conditions of 5.5 mM glucose for 4 days, 10–1000 nM linagliptin had no influence on β -cell turnover (data not shown). Elevated glucose concentrations, free fatty acids, cytokines, and oxidative stress induced β -cell apoptosis (2.5-, 3.6-, 3–1-, 3.7-, and 4.5-fold induction by 11.1 and 33.3 mM glucose and by 0.5 mM palmitate, IL-1 β +IFN- γ , and H_2O_2 , respectively, $P < .001$, Figure 1A) in isolated human islets, compared with control incubations. This was reversed by pretreatment with IL-1Ra as well as with linagliptin. IL-1Ra reduced apoptosis 2.5-, 2.0-, 2.1-, and 3.0-fold in 11.1 and 33.3 mM glucose, palmitate, and IL-1 β +IFN- γ , respectively ($P < .01$), but in H_2O_2 , only a tendency but no significant

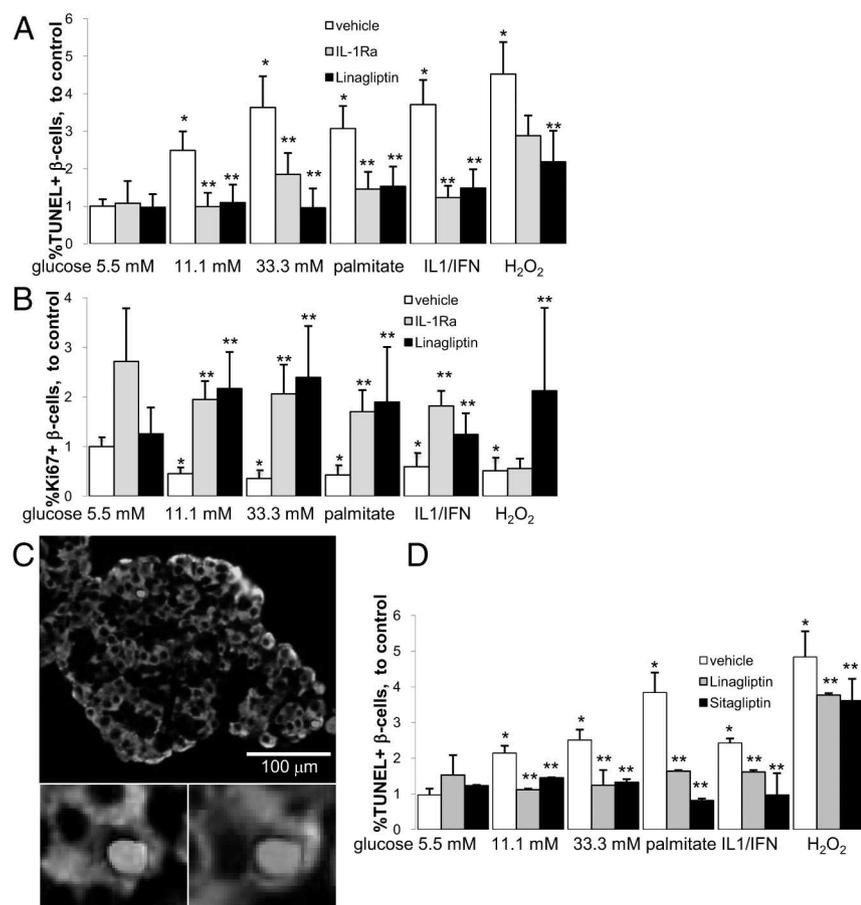


Figure 1. DPP-4 inhibition improves β -cell survival. Human pancreatic islets were cultured on ECM-coated dishes (A and B) or in suspension (C and D) at 5.5 mM (control), 11.1 or 33.3 mM glucose, 0.5 mM palmitate, or 2 ng/mL IL-1 β per 1000 IU IFN- γ for 96 hours (A and B) or 72h (C and D) or at 50 μ M H₂O₂ for 8 hours (A–D) in the absence (control) or presence of IL-1Ra (500 ng/mL) or linagliptin (50 nM). Apoptosis was analyzed either directly in the dishes (A) or in paraffin-embedded islet sections (C and D: nuclei) by the TUNEL assay. Proliferation was analyzed in the dishes (B) by the Ki67 antibody. All specimens were double stained for insulin. Results are means \pm SEM of the TUNEL- or Ki67-positive β -cells, normalized to control conditions at 5.5 mM glucose (in absolute numbers: 0.4% TUNEL and 0.37% Ki67 positive β -cells at 5.5 mM glucose on the extracellular matrix and 1.0% TUNEL positive β -cells at 5.5 mM glucose in suspension culture). The average number of β -cells counted was 4175 for each treatment group from 3 different experiments from 3 different organ donors. C, A representative staining picture of a palmitate-treated human islet (upper panel, \times 200 magnification) with 2 TUNEL-positive nuclei (lower panel, \times 600 magnification). *, $P < .05$ compared with vehicle-treated control at 5.5 mM glucose; **, $P < .05$ compared with vehicle under the same treatment condition.

protective effect was observed (Figure 1A). Fifty nanomoles of linagliptin reduced apoptosis 2.3-, 3.8-, 2.0-, 2.5-, and 2.1-fold in all diabetogenic conditions ($P < .01$, Figure 1A).

Human islets on the ECM-coated dishes had a very limited capacity to proliferate in culture (0.4% \pm 0.1% Ki67/insulin double positive cells at 5.5 mM glucose basal condition). A diabetic milieu even further reduced this proliferative capacity (2.2-, 2.8-, 2.3-, 1.7-, and 1.9-fold reduction by 11.1 and 33.3 mM glucose and by 0.5 mM palmitate and IL-1 β +IFN- γ when incubated for 4 days and H₂O₂ when incubated for 8 hours, respectively, compared with control, $P < .01$, Figure 1B). In contrast, IL-

1Ra and linagliptin restored proliferation (4.3-, 5.8-, 4.0-, and 3.1-fold induction by IL-1Ra and 4.8-, 6.8-, 4.4-, 2.1-, and 4.1-fold by linagliptin in 11.1 and 33.3 mM glucose, in 0.5 mM palmitate, IL-1 β +IFN- γ , and H₂O₂, respectively, compared with vehicle, $P < .01$, Figure 1B). IL-1Ra had no protective effect on β -cell proliferation under H₂O₂ exposure. This was also confirmed in mouse islets, which were exposed for 8 hours to 50 μ M H₂O₂ with or without 500 ng/mL IL-1Ra. Apoptosis was induced 3.6-fold by H₂O₂, whereas IL-1Ra failed to show any protective effect (data not shown).

To exclude a matrix-dependent effect, we cultured isolated human islets in suspension culture dishes, exposed them for 3 days or 8 hours (H₂O₂) to the above-described diabetogenic milieu and analyzed β -cell apoptosis in Bouin-fixed, paraffin-embedded islet sections. A similar induction of β -cell apoptosis was observed by all diabetogenic culture conditions, and linagliptin significantly reduced β -cell apoptosis (1.9-, 2.0-, 2.3-, 1.5-, and 1.2-fold in 11.1 and 33.3 mM glucose, 0.5 mM palmitate, IL-1 β +IFN- γ , and H₂O₂, compared with vehicle, $P < .01$; Figure 1D). Sitagliptin, another DPP-4 inhibitor tested in the same experiments, had similar protective effects (Figure 1D; 1.4-, 1.9-, 4.7-, 2.5-, and 1.3-fold reduction of β -cell apoptosis in 11.1 and 33.3 mM glucose, 0.5 mM palmitate, IL-1 β +IFN- γ , and

H₂O₂, $P < .01$).

Linagliptin improves β -cell function and inhibits oxidative stress

In addition to its protective effect on β -cell survival, linagliptin improved GSIS in islets after long-term culture of 4 days. In control conditions, there was a 4-fold stimulatory index when glucose was increased from 2.8 mM to 16.7 mM glucose (Figure 2D). All diabetogenic culture conditions significantly reduced GSIS ($P < .01$; Figure 2, A and D). At basal glucose, neither IL-1Ra (Figure 2, B and D) nor linagliptin (Figure 2, C and D) increased the stim-

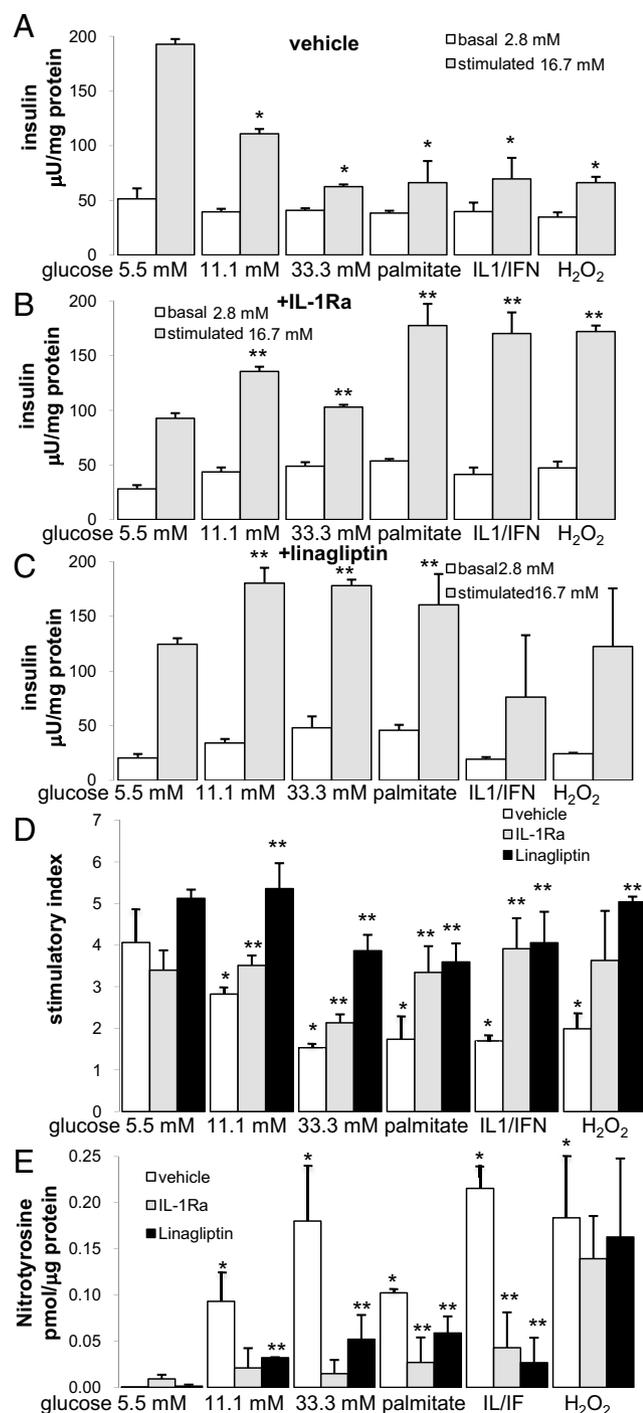


Figure 2. Linagliptin improves β -cell function and inhibits oxidative stress. Human pancreatic islets were cultured on ECM-coated dishes at 5.5 mM (control), 11.1 or 33.3 mM glucose, 0.5 mM palmitate, or 2 ng/mL IL-1 β per 1000 IU IFN- γ for 96 hours or at 50 μ M H₂O₂ for 8 hours in the absence (control; A and D) or presence of IL-1Ra (500 ng/mL; B and D) or linagliptin (50 nM; C and D). Basal and stimulated insulin secretion indicates the amount secreted during 1-hour incubations at 2.8 (basal) and 16.7 mM (stimulated) glucose after the culture period and normalized to protein content (A–C). The stimulatory index was calculated as stimulated/basal insulin secretion (D). Nitrotyrosine content (E) in the islet lysates was normalized to whole-islet protein content. Results are means \pm SEM from triplicates from 3 independent experiments from 3 donors, *, $P < .05$ compared with vehicle-treated control at 5.5 mM glucose; **, $P < .05$ compared with vehicle under the same treatment condition.

ulatory index, but under the diabetogenic conditions, GSIS was highly improved (1.2-, 1.4-, 1.9-, 2.3-, and 1.8-fold by IL-1Ra, Figure 2, B and D, and 1.9-, 2.5-, 2.1-, 2.4-, and 2.5-fold by linagliptin, $P < .05$, Figure 2, C and D).

To investigate the underlying mechanisms of the protective effect of the DPP-4 inhibitor, we hypothesized that changes in nitrotyrosine, a marker for oxidative stress, may occur during the long-term exposure with diabetogenic conditions. Nitrotyrosine production in islets during the 4-day culture period was measured and normalized to whole protein content of the islet lysate. The nitrotyrosine content was increased by glucose, palmitate, cytokine, and H₂O₂ exposure ($P < .05$; Figure 2E). Both IL-1Ra and linagliptin decreased nitrotyrosine under glucose, palmitate, and cytokine treatment ($P < .05$) but not under conditions of H₂O₂ (Figure 2E).

Linagliptin stabilizes GLP-1 in the supernatants of human islet cells

Because the main effect of DPP-4 is to stabilize GLP-1, we next investigated whether total and active GLP-1 levels in the culture medium were increased by linagliptin treatment. Diabetogenic conditions (increasing glucose concentrations and cytokines) induced accumulation of total GLP-1 (Figure 3A) in the islet supernatants, and linagliptin (30 nM) pretreatment resulted in an approximately 2-fold higher total GLP-1 (Figure 3A) and an approximately 3-fold higher active GLP-1 (Figure 3B) in all conditions. Levels of total and active GLP-1 were unchanged by IL-1Ra (data not shown).

Next, the effect of linagliptin on the stabilization of an exogenously added GLP-1 concentration was investigated. One hour after linagliptin (30 nM) treatment, 0.5 nM GLP-1 (a concentration similar to the secreted total GLP-1 from the islets treated with linagliptin) was added to the islet culture medium and immediately measured. Rapid degradation of GLP-1 was observed during the culture (Figure 3, C and D), whereas the addition of linagliptin inhibited such degradation of GLP-1. The total (Figure 3C) and active GLP-1 (Figure 3D) accumulated during culture in the linagliptin-treated islet supernatants, which did not occur in the nontreated islets.

GLP-1 stabilization was accompanied by restored GLP-1 receptor levels. In confirmation with earlier studies (30, 31), GLP-1 receptor levels were decreased in response to glucotoxicity and cytokine exposure, compared with untreated control. In contrast, 30 nM linagliptin increased GLP-1 receptor levels in all diabetogenic conditions (Figure 3E). To further prove whether the effect of linagliptin is GLP-1 dependent, we pretreated linagliptin with exendin-(9–39), an established inverse agonist of the GLP-1 receptor (32, 33). Although 30 nM linagliptin was effec-

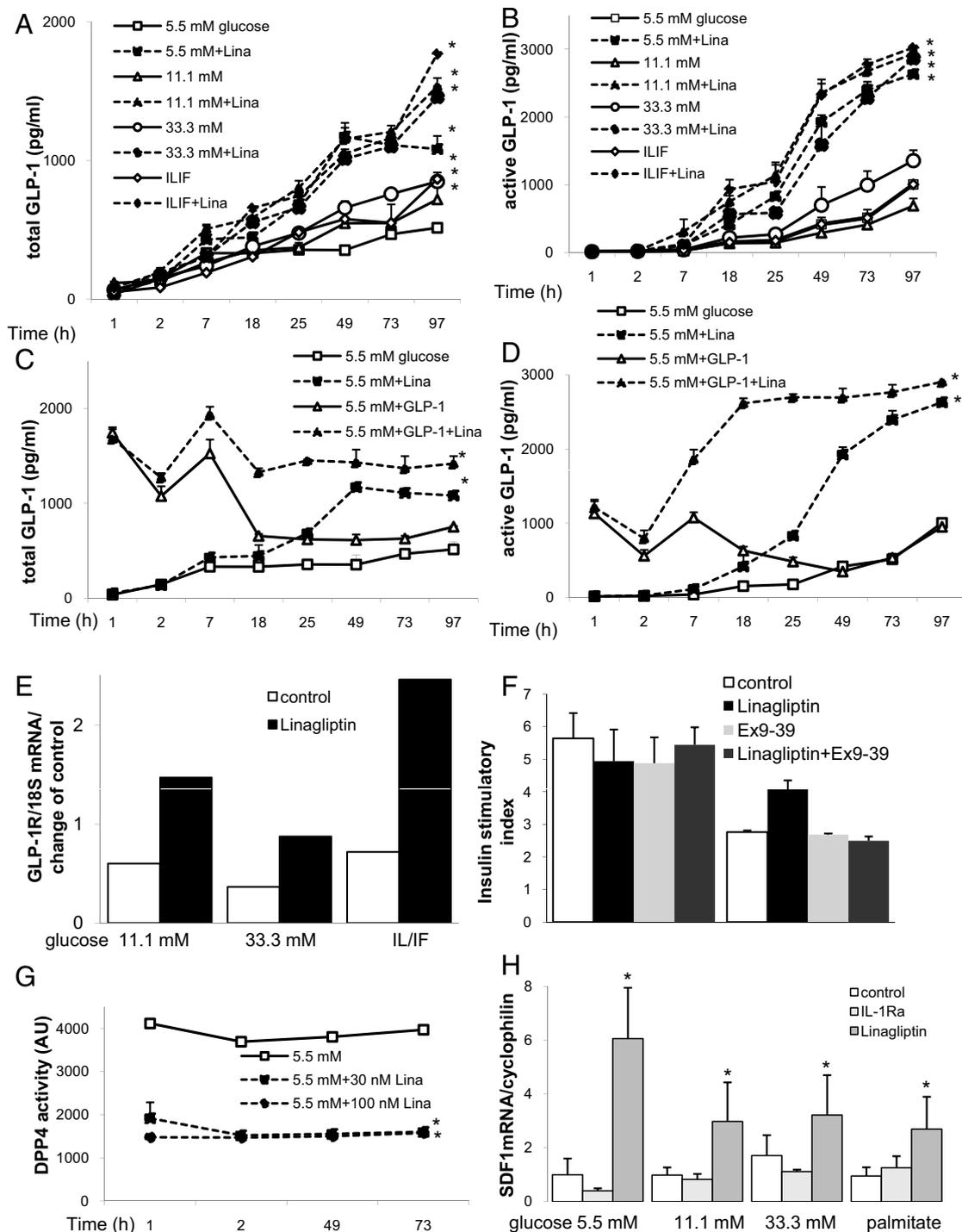


Figure 3. Linagliptin stabilizes GLP-1 in the supernatants of human islet cells. Human pancreatic islets were cultured on extracellular matrix-coated dishes at 5.5 mM glucose (control) or 11.1 and 33.3 mM glucose, 0.5 mM palmitate, or 2 ng/ml IL-1 β per 1000 IU IFN- γ for 96 hours in the absence (control) or presence of linagliptin (50 nM; A and B) or 0.5 nM GLP-1 (C and D). Total (A and C) and active (B and D) GLP-1 secretion was measured in the supernatants during the 4-day culture period. mRNA was isolated from treated islets after 4 days, and RT-PCR analysis of the GLP-1 receptor levels (E) was performed. The levels of gene expression were normalized to tubulin and 18S and presented as the change of 5.5 mM glucose control. Human pancreatic islets were cultured at 5.5 mM (control) glucose or 2 ng/mL IL-1 β per 1000 IU IFN- γ for 96 hours in the presence of 30 nM linagliptin and an inverse agonist of the GLP-1 receptor exendin-(9–39) (100 nM; F). The insulin stimulatory index as the ratio of the stimulated and basal insulin secretion was calculated. G, DPP-4 activity was measured at control conditions and with 30 and 100 nM linagliptin during culture. H, mRNA was isolated from treated islets after 4 days of culture and RT-PCR analysis of SDF1 α performed. The levels of gene expression were normalized to tubulin and cyclophilin (with similar results) and presented as change of control. Results are means \pm SEM from triplicates from 3 independent experiments from 3 donors, except for E [data are from 1 single donor (in triplicates)] and F [from 2 donors (each in triplicates)]. *, $P < .05$ compared with vehicle-treated control at 5.5 mM glucose.

tive in improving GSIS under cytokine exposure, the addition of 100 nM exendin-(9–39) inhibited the linagliptin effect (Figure 3F). To prove that linagliptin was active, we also measured DPP-4 activity in the islet cell supernatants. As expected, linagliptin inhibited DPP-4 activity in culture (Figure 3G). The potential GLP-1-SDF1 α synergism strongly promotes β -cell survival (34). Because SDF1 α is another substrate of DPP-4 (35) and an important pro-survival factor for β -cells (36), we tested whether the increases in GLP-1 concentration and the reduction in DPP-4 activity by linagliptin are also paralleled with SDF1 α mRNA production. Linagliptin strongly increased SDF1 α under basal as well as under diabetogenic conditions ($P < .05$, Figure 3H), whereas glucose and palmitate themselves or IL-1Ra treatment of the islets had no such effect on SDF1 α (Figure 3H).

Linagliptin inhibits cytokine secretion and production from islets under diabetogenic conditions

Because proinflammatory cytokines and chemokines are elevated under a diabetogenic milieu, we tested whether IL-1Ra and linagliptin exert their protective effects through an inhibition of cytokine secretion. Cytokine and chemokine secretion into the culture medium was measured simultaneously by the Meso Scale Discovery technique and mRNA production by RT-PCR. The diabetogenic milieu, which included elevated glucose concentrations and palmitate, significantly increased IL-1 β secretion (Figure 4A) and mRNA production (Figure 4B), whereas IL-6 (Figure 4, C and D) and IL-8 (Figure 4, E and F) were increased by the cytokine mixture IL-1 β and IFN- γ , but no significant effect was observed in the other diabetogenic conditions. Although IL-1Ra and linagliptin had no effect on the minimal basal IL-1 β secretion and production during the 4-day culture period, they significantly decreased both IL-1 β secretion and mRNA production (Figure 4, A and B) under diabetogenic conditions. Similarly, IL-6 and IL-8, which were elevated in response to IL-1 β /IFN- γ , were decreased by IL-1Ra and linagliptin (Figure 4, C–F).

Discussion

Orally administered DPP-4 inhibitors prevent the rapid cleavage of GLP-1 and thus increase levels of active GLP-1, resulting in increased insulin and reduced glucagon secretion, lowered glucose, and reduced glycosylated hemoglobin levels by 0.5%–1.0% (2). Linagliptin improves glycemic control both as monotherapy (37) and in

combination therapy, eg, with metformin (38) or pioglitazone (39), and also improves insulin sensitivity (39). DPP-4 inhibitors mimic many of the actions ascribed to GLP-1 in rodents including preservation of β -cell mass through stimulation of cell proliferation and the inhibition of apoptosis in rodents (15–17).

In addition to glucose-dependent insulinotropic polypeptide and GLP-1, many gastrointestinal hormones, neuropeptides, cytokines, and chemokines are substrates for DPP-4 (40), including C-X-C motif chemokine 10 (CXCL10) and SDF1 α , both of which are involved in immune regulation and influence the inflammatory response and have been associated with the regulation of β -cell turnover in diabetes (23, 34). Because cytokines and chemokines are crucial for the survival of pancreatic β -cells (41), it remained to be investigated whether DPP-4 inhibitors in culture directly affect β -cell survival and function.

In the present study, the DPP-4 inhibitor linagliptin effectively restored proliferation, survival, and β -cell function in a diabetic milieu. The concentrations of 30–50 nM we used were higher than the average maximum concentration plasma levels but also could be obtained in patients taking therapeutic doses of linagliptin (42). At all concentrations up to 1 μ M, which is 1000-fold the IC₅₀ of the human enzyme, linagliptin did not affect β -cell survival at basal conditions in culture.

Human β -cells in culture have only a very limited, if any (42), capacity to proliferate. Here we found 0.4% proliferating β -cells under basal conditions when islets were plated on ECM-coated dishes, and proliferation was further decreased by diabetogenic conditions of chronically elevated glucose, free fatty acids, cytokines, or H₂O₂. Linagliptin improved cell survival by decreasing apoptosis and maintained cells at a steady state level, even under diabetogenic conditions.

The protective linagliptin effect seen in this study was mediated by the stabilization of GLP-1 in the islet supernatants, in which total as well as active GLP-1 levels were restored and accumulated during islet culture, providing a similar mechanism as seen in vivo.

Active GLP-1 can be produced from islets (43). The α -cells express prohormone convertase 1/3, which cleaves proglucagon peptide to generate GLP-1. α -Cells are an established islet source of secreted GLP-1 (44–47) and may provide a local protective paracrine effect for β -cell regeneration under diabetic conditions, especially during β -cell injury (34). Sorted human β -cells also express prohormone convertase 1/3, but the GLP-1 content could not be detected, and thus, it was assumed that β -cells do not produce GLP-1 (48), although it was found to be secreted from the rodent β -cell line, INS-1E (49).

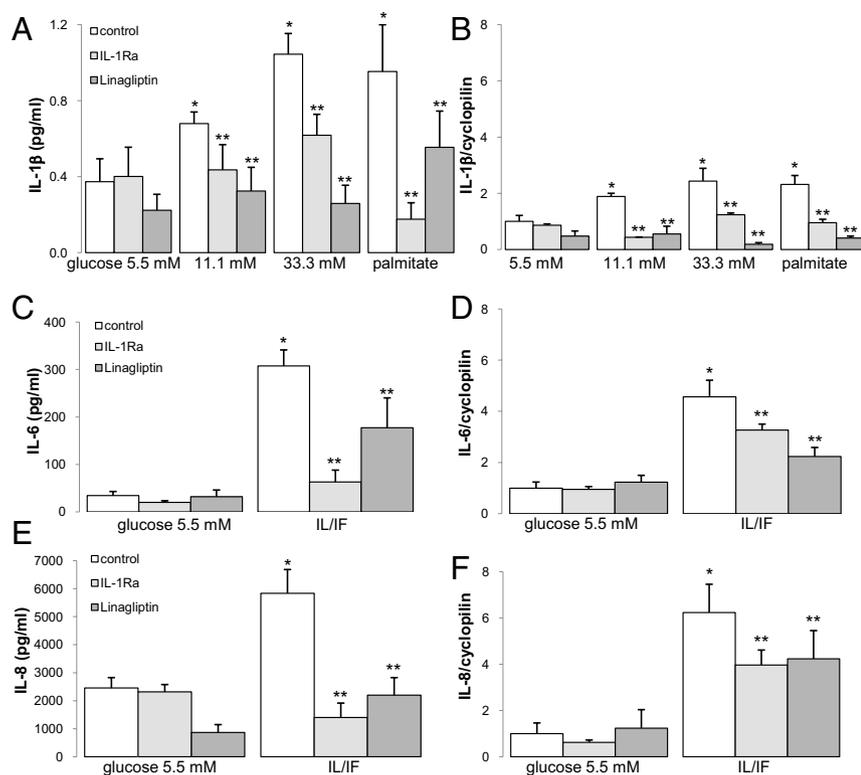


Figure 4. Linagliptin inhibits cytokine secretion from islets under diabetogenic conditions. Human pancreatic islets were cultured on extracellular matrix-coated dishes at 5.5 mM (control), 11.1 or 33.3 mM glucose, 0.5 mM palmitate, or 2 ng/ml IL-1 β per 1000 IU IFN- γ for 96 hours in the absence (control) or presence of IL-1Ra (500 ng/mL) or linagliptin (50 nM). B, D, and F, mRNA was isolated from treated islets after 4 days and RT-PCR analyses of IL-1 β (B), IL-6 (D), and IL-8 (F) were performed. The levels of gene expression were normalized to tubulin and cyclophilin (with similar results) and presented as change of control. Cytokine and chemokine secretion of IL-1 β (A), IL-6 (C), and IL-8 (E) from the culture medium of the same islets were analyzed in a multiplex assay (Meso Scale Discovery). Results are means \pm SEM from 3 independent experiments from 3 donors. *, $P < .05$ compared with vehicle treated control at 5.5 mM glucose; **, $P < .05$ compared with vehicle under the same treatment condition.

We found high levels of active GLP-1 (~ 3 ng/mL from 50 islets over 4 days of culture) were secreted from human islets and accumulated only in the presence of a DPP-4 inhibitor, whereas high DPP-4 activity could be measured only in the absence of linagliptin.

GLP-1 is accumulated in the medium during the 4-day culture period and provides in turn a paracrine protective effect on the β -cell. Diabetogenic conditions, eg, the cytokine cocktail IL-1 β /IFN- γ , enhanced IL-6 production. This was counteracted by linagliptin and paralleled with increased GLP-1 in the medium. It can thus be hypothesized that higher GLP-1 concentrations in the culture medium in turn inhibits IL-6 production; this would provide a negative feedback loop to the IL-6 effect to induce GLP-1 in islets (44).

Hyperglycemia and a diabetic milieu decrease expression of β -cell GLP-1 receptors in vivo and in vitro (30, 31, 50). In contrast, diabetogenic conditions significantly increased total GLP-1 release from cultured islets. This was also observed in islets from patients with T2D (45) and

from diabetic *Psammomys obesus* (47), which show higher GLP-1 secretion than nondiabetic controls.

Slightly reduced GLP-1 secretion after a meal in patients with impaired oral glucose tolerance and more severely impaired GLP-1 secretion in type 2 diabetic patients was observed (52), although controversial results have been obtained in various studies (53). Differences occur between tissue-specific GLP-1 levels and whether total or active GLP-1 was measured (54). It is possible that reduced GLP-1 in T2D patients is the result of elevated plasma DPP-4 activity (55), which can be induced by chronic hyperglycemia and is paralleled with reductions of active GLP-1 (56).

Linagliptin strongly reduced DPP-4 activity in the human islet supernatant and stimulated GLP-1 stability. Active DPP-4 rapidly degrades SDF1 α (1–68) to inactive SDF1 α (3–68) (57), and thus, more active SDF1 α is available upon DPP-4 inhibition. In turn, SDF1 α acts like a cytokine and induces its own production (34). This explains the increased SDF1 α mRNA levels with linagliptin treatment, found in the present study.

The regulation of DPP-4 in diabetes has not been fully clarified, and both increased (55, 56) and decreased (58) DPP-4 activity has been reported in patients with diabetes. DPP-4 release from adipose tissue is higher in obese individuals and correlates with parameters of the metabolic syndrome, in which DPP-4 directly impairs insulin signaling in fat and muscle cells (59). Elevated glucose exposure leads to enhanced DPP-4 activity and mRNA expression in endothelial cells (51). Also, in human islets, we detected elevated DPP-4 mRNA in response to elevated glucose and palmitate, whereas linagliptin inhibited this induction (data not shown). One could speculate that in islets under diabetogenic conditions, there is more DPP-4 produced, which inactivates the elevated GLP-1, and thus, it cannot fulfill its functions.

Recent human studies investigating the long-term treatment with vildagliptin on β -cell function were somehow disappointing because after the washout periods, the observed β -cell-sparing effects disappeared, thus questioning

the disease-modifying potential of this drug (6). However, it remains to be elucidated whether this is caused by a limited tissue penetration in vivo or limited availability of the drugs in islets.

In summary, we show that linagliptin has a protective effect on β -cell turnover and function under diabetogenic conditions, which is mediated through stabilized GLP-1 and an antiinflammatory/antioxidative stress pathway.

Acknowledgments

We thank Jennifer Bergemann (Uni Bremen), Katja Thode, Julia Dennenmoser, and Annette Halder (Boehringer Ingelheim) for excellent technical assistance. Human islets were provided through the JDRF Award 31-2008-413 (European Consortium For Islet Transplantation Islet for Basic Research program).

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This work was supported by the German Research Foundation (DFG, Emmy Noether Programm MA4172/1-1) and the European Research Council.

Disclosure Summary: T.K. and D.M.S. are employees of Boehringer Ingelheim Pharma. Linagliptin is a Boehringer Ingelheim Pharma product. There are no patents, products in development, or other marketed products to declare. All of the other authors have nothing to disclose.

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Acknowledgements

I took up biology in college simply out of naive love for medicine not knowing where it would exactly lead but it looks like a good place right now! Thus, going on from biology to microbiology to biochemistry, here I ended up with Kathrin, trying to cure diabetes ;)

So, that's precisely where I would start, thanking one of the most cheerful and enigmatic person I have known. Ambitious, energetic and fit as hell! It's been a journey of 6 years with you, since the lab rotation in 2009. I must admit your speed has been a tad bit intimidating – with always prompt e-mail replies to simply running through corridors or the entire university for that matter...you actually had one of those small bicycles to get to places faster! Though I could never possibly match that speed I'm glad some of our interests matched when you took the leap with me to look at islet vascularisation. It's definitely been a roller-coaster ride with all the uncertainties and doubts but considering I've reached this stage of my thesis, I guess we pulled through. It's been an excitingly bumpy ride with you Kathrin – all the Thanks-giving dinners, Haus am Walde parties, conferences and or simply chats during islet isolations. Thanks a lot for the opportunity to be here and for mentoring me!

My PhD can now be officially dived into 2 parts – pre-gita and post-gita. From being my lab rotation supervisor to a roommate to a best friend! That indeed is a long journey we made and I'm glad there's no end to it anytime soon. A-B-C to science, we've got it all covered. You literally turned the tearful times to smiles! Thanks for making not just lab-life but Bremen-life one hell of a fun ride!

Thanks to Amin and Federico for welcoming me to the lab by all bullying during the lab rotation. What a riot it was...while we were a small group ;) Ardestani!!! I'm definitely going to miss the silent conversations sitting diagonally across the office desk...so you were always there! Not just across the office and every single Sunday and motivating me...basically being the big Daddy! Federico I would miss probably with every cup of coffee :D I think we'll stay connected until we have the common God – Clapton between us! A scientist, musician, artist and absolute delight to be with...I think you've been the most dynamic part of the group...and I hope you continue to not only watch the wheels turn round and round but stay chilled out and enthusiastic in every walk of life. Thanks Zahra – for always being the sugar candy

around and adding a special dimension to the tea/lunch time. Jenny-Fussball, Weihnachtst kuchen, und leckere pasta salad! Thanks for always helping out with tons of things outside the lab and within and for the sneak peek to deutsches kultur. I also want to thank Niels for helping with some of the non-scientific troubles and sharing student woes lately! Apart from Jenny, I'd like to also thank all other ex-lab members who have been a part of atleast some time of my journey here – Erna, Katrin and Katharina.

I would especially like to thank all the current members of the lab – Katrischa (for all the help with mice), Anke (Thanks for always being so willing to help!), Wei, Ting, Durga, Karthika, Michael, Sahar, Lei and Delsi.

I'm very grateful to P-O Carlsson for agreeing to review my thesis, collaborating and guiding me though the project.

Thanks to Prof. Kelm for being the examiner in the committee.

Well, that's about it in the lab but life as an expat and thus the PhD has been so much more than just this. My ex-roommates Jui and Teja! I wouldn't have survived in that shady WG without you guys..thanks for making it a home. Minu you're a rock! A gem of a person and a best friend...from the bachelors in India right upto PhD completion...life in Germany was another added basket of fun with you around.

Moving on across the globe, to the west - Maddy, Kamu, Esh (east), Anuja and Dips for keeping me going every single Sunday with our legendary conference calls! Friends truly are family away from home 😊

And lastly, the people who are my lifeline and I truly am what I am because they exist and support and love – Mom, Dad, Dida and Jiju!